IN VIVO AND IN VITRO DEVELOPMENT OF THE GENICULOCORTICAL PATHWAY IN THE MOUSE

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DECLARATION

I declare that this thesis was composed by myself. Contributions of others to the work are clearly indicated.

QUOTE

"In research the horizon recedes as we advance,
and is no nearer at sixty than it was at twenty.
As the power of endurance weakens with age,
the urgency of the pursuit grows more intense.
.....And research is always incomplete .

MARK PATTISON

1813-1884

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ABSTRACT

This is an investigation of the development of connections between the lateral geniculate nucleus (LGN) and the visual cortex in the mouse, using both in vitro and in vivo techniques.

Visual cortical layer 4 is the target of ingrowing geniculate axons. Using immunohistological labelling techniques, I monitored the pre and postnatal migration of cells destined for layer 4 from the ventricular zone to the cortical plate in vivo. It was shown that, although the first of these cells enter the cortical plate as early as embryonic day 16 (E16), migration continues and the majority of layer 4 cells are in place by postnatal day 3 (P3), however migration is still occurring until after P7.

The development of connections between the LGN and the visual cortex was investigated using in vivo immunohistological and tract-tracing methods with the carbocyanine dye, DiI. The birth of the LGN is complete by E14 and geniculocortical fibres are present in the visual cortex by E17.

The main emphasis of this study was the mechanisms which control the outgrowth of axons from the LGN. Using an organotypic co-culture system, I searched for evidence of long range growth-promoting or trophic interactions between LGN explants and the occipital cortex. This technique has been widely used, with great success, by Yamamoto et al. (1989 and 1992), Bolz et al. (1990), Molnar and Blakemore (1991) and Toyama et al. (1991) to investigate the growth of axons into the visual cortex. In this study I have investigated the outgrowth of neurites from LGN explants before they came into contact with the target explant. Blocks of embryonic LGN were cultured in serum-free medium, either alone or with slices of embryonic cortex or early postnatal occipital cortex, frontal cortex, cerebellum, medulla or liver (the ages of the explants

were selected on the basis of the previous in vivo study). When the LGN explants were cultured either alone or with liver, little or no outgrowth was observed, whereas in the presence of occipital cortex there was profuse geniculate outgrowth. The frontal cortex, cerebellum and medulla all stimulated significantly less outgrowth than the occipital cortex. The main effect on outgrowth was on neurite density rather than neurite length. These findings suggest that several regions of the brain release factor(s) that can stimulate outgrowth from LGN explants, but the occipital cortex is the most effective.

Using a series of conditioned medium experiments, I confirmed that the factors produced by the occipital cortex were freely diffusible in the culture medium. There was significantly more outgrowth from LGN explants cultured alone in conditioned medium than in control medium. To test if the factors were having a trophic effect I cultured blocks of LGN with and without occipital cortex and then prepared them for light and electron microscopy. There was no difference between the proportions of healthy and pyknotic cells in LGN explants that had been cultured alone and those that had been cultured with occipital cortex, indicating that the factors were not required for the survival of embryonic geniculate cells. Nerve growth factor (NGF) was added to control medium, at various concentrations, to see if the outgrowth observed from the LGN explants cultured with occipital cortex and in conditioned medium could be mimicked. NGF had no effect at low concentrations and only a slight effect at very high concentrations, and so is unlikely to be the factor released by occipital cortex.

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CHAPTER 1

INTRODUCTION

1.1 *DEVELOPMENT OF THE BRAIN*

The major events which occur during the development of the brain are well documented. However, the fine tuning of this system and the mechanisms which control its development are not as well understood.

The brain emerges from three prominent swellings at the anterior end of the neural tube. These swellings represent the three main parts of the brain; the forebrain, the midbrain and the hindbrain. In recent years, attention has centred on how the many constituent parts of these areas are generated, the mechanisms which control the migration of the cells within them, how cells "know" when they have reached their final positions and how neurons in different, and often distant, parts of the brain make specific connections with each other.

1.1.1 The importance of studying development

Development is the term assigned to the process by which all organisms are generated. Development begins with the first divisions of a cell after fertilisation. Our quest to understand the events which occur during development is driven by our need to increase our medical knowledge and our ability to treat patients when a small part of the process goes wrong. Before we can address and attempt to resolve the many problems which occur as a result of the failure of even a very small part of the developmental process, we require a comprehensive knowledge of the events which occur normally.

1.2 *THE STUDY OF THE VISUAL SYSTEM*

This thesis addresses the development of the brain and in particular, the visual system. The visual system is composed of many different levels of processing. This thesis is centred on the development of the pathway from the lateral geniculate nucleus (LGN) to the visual cortex.

The mouse was chosen for this study for several reasons. Firstly, it is easily available, and it has a short gestation period. Secondly, there are potential uses of the species for future genetic studies, in view of the well characterised genetic mutations such as reeler (where the cortical layers are inverted) and the possibility of transgenic work.

1.3 *THE ANATOMY OF THE ADULT VISUAL SYSTEM*

The anatomy of the visual system, although complex, is well understood. To better understand the development of constituent parts of the visual system, we must first become familiar with the anatomy of this system as a whole. The neural response to light begins in the retina, and then progresses through the LGN to the visual area of the cerebral cortex.

1.3.1 The retina

At the first level in the visual pathway, the retina, there is transduction of light into neural activity (see e.g. Michael, 1969). The retina is highly organised, with all vertebrate retinae constructed using the same basic plan. The retina is composed of 3 nuclear (cellular) layers: the ganglion cell layer (for more information on this layer see Maturana et al., 1960; Stell and Witkovsky, 1973; and Boycott and Wassle, 1974), the inner nuclear layer and the outer nuclear layer. These layers are seperated by 2 plexiform (synaptic) layers: the inner plexiform layer and the outer plexiform layer (Ramon Y Cajal, 1911).

The inner nuclear layer contains the cell bodies of 3 neuronal cell types: the horizontal cells, the bipolar cells, and the amacrine cells. The horizontal cells transfer information laterally within the outer plexiform layer, while the amacrine cells transfer information laterally within the inner plexiform layer. The bipolar cells transfer information from the photoreceptors to the ganglion

cells (e.g. Dowling and Dublin, 1989).

The outer nuclear layer contains the photoreceptor cells, the rods and cones, which are responsible for the transduction of light (Cohen, 1972). One type of glial cell is also present in the retina: the Muller cells. These cells extend vertically through the entire retina. Their cell bodies are situated mainly in the inner nuclear layer. This brief summary covers the major features of the anatomy of the retina, as described in more detail by for instance Cajal (1911), Polyak (1941), Kuffler (1953), Dowling and Boycott (1966), Boycott and Dowling (1969) and Stell (1972).

Light passes through the optics of the eye and enters the retina through the ganglion cell layer. It then penetrates the entire thickness of the retina and is absorbed by the photoreceptors, where it is transduced. The resultant activity is transferred first to the bipolar cells then to the ganglion cells. The ganglion cell axons form the optic nerve which projects into the brain terminating on a relay nucleus, the LGN, in the thalamus.

Light from the left half of the visual field is processed in the right half of each retina, and is relayed exclusively to the right visual cortex. Light from the right half of the visual field is processed in the left half of each retina, and is relayed exclusively to the left visual cortex (see Figure 1 to clarify these points).

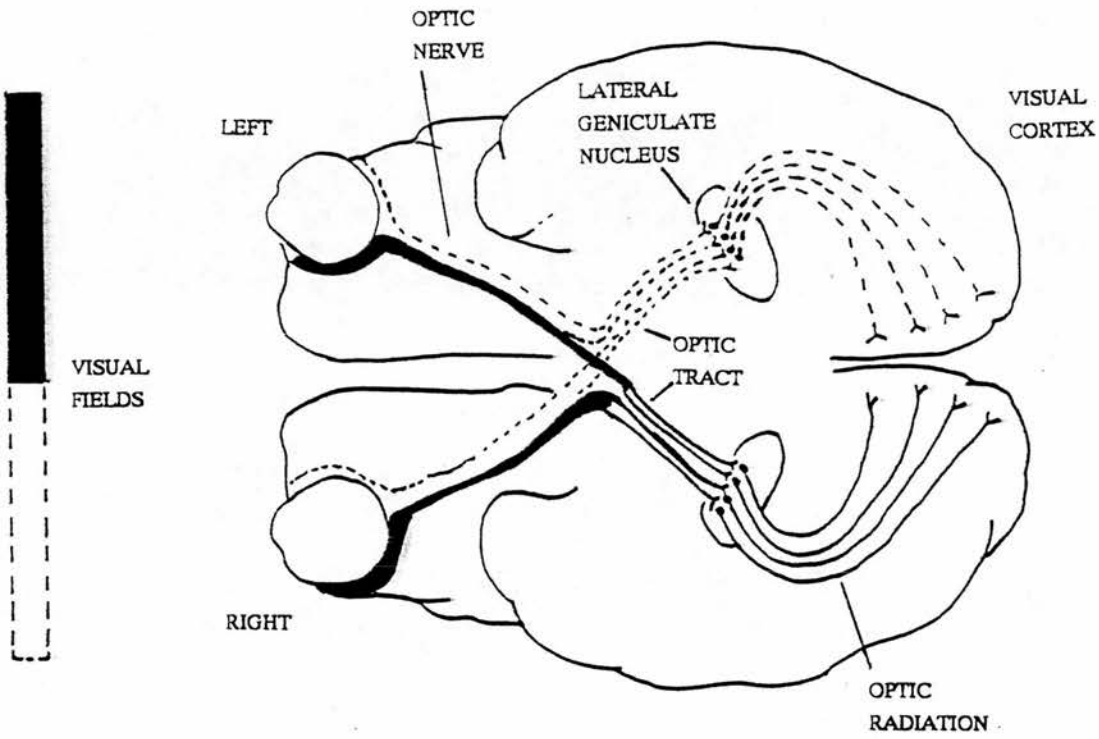


Fig. 1 Schematic diagram of the visual pathway in primates. The left side of each retina projects to the left LGN, which projects to the left visual cortex. The left visual cortex receives its information from the right side of the visual field only. (Modified from Kuffler, Nicholls and Martin, 1984).

1.3.2 The lateral geniculate nucleus (LGN)

The thalamus is one of the main relay centres of the brain and is composed of many discrete nuclei, each concerned with a different sensory pathway. The LGN is one of these nuclei.

The LGN is a discrete layered structure. The monkey LGN has 6 layers, numbered 1-6. It has been shown anatomically that each layer receives input from one or the other eye. Layers 1, 4 and 6 receive input from the contralateral eye (eye on the opposite side), while layers 2, 3 and 5 receive input from the ipsilateral eye (eye on the same side). In the cat, the LGN has 3 distinct layers of cells; A, A_1 and C, with C being a very complicated layer and therefore further subdivided (e.g. Guillery, 1970). Layers A and C receive input from the contralateral eye, while A_1 receives input from the ipsilateral eye. The rodent LGN has two distinct regions, shown using anatomical methods (Cunningham and Lund, 1971). These regions, however, are not as well defined as in either monkey or cat. The lateral-posterior region is similar to layer A of the cat, while the anteriomedial region resembles cat layer C.

Segregation of input to the different layers of the LGN in the adult has been shown both anatomically and electrophysiologically. Terminals from the contralateral eye are confined to the layers supplied by that eye, with the same true of the ipsilateral eye. There is little spill-over across the border of layers (Guillery, 1970; Rakic, 1977; Bowling and Michael, 1980; Shatz, 1983). In higher species the LGN is layered to accommodate the different functional properties of inputs from the retina. For example, layer 4 cells of monkey LGN respond to different colours of light, while layers 1 and 2 respond to moving stimuli (Schiller and Malpeli, 1978; Kaplan and Shapely, 1982).

The output from the LGN to the visual cortex originates from the thalamocortical cells. The axons of these cells project through the optic

radiation to the cerebral cortex, where they form synapses with cells of layer 4 and some on layer 6 cells. Axons terminating in layer 6 may be branches of axons projecting to layer 4.

Thalamic nuclei which project to the visual cortex receive reciprocal connections from the cortex. Thus, the LGN receives a second type of input which originates from the pyramidal cells of visual cortical layer 6. These are termed the corticothalamic fibres (e.g. Jacobson and Trojanowski, 1975; Peters and Saldanha, 1976; Sefton et al., 1981).

1.3.3 The cerebral cortex: visual areas

The cerebral cortex is a six-layered structure with layer 1 being the most superficial, situated immediately below the pial surface, and layer 6 the deepest, found immediately above the white matter (e.g. Cajal, 1911; Szetagothai, 1969; Rakic, 1974). The different layers can be distinguished by their cellular content. Layer 1, known as the molecular layer, is composed of a small number of cells, known as Cajal-Retzius cells, but mainly of fibres and apical dendrites arising from the cells of deeper layers. Layers 2 and 3 are composed mainly of superficial pyramidal neurons (termed pyramidal neurons due to the shape of the cell body) with a small number of bipolar cells. Layer 4 is composed of smaller stellate/granule cells. Layers 5 and 6 are composed of deep pyramidal cells; although these cells have a similar shape to those found in layers 2 and 3, the cell body is larger.

The visual cortex is subdivided into distinct regions or areas. In the monkey there are at least 10 to 15 visual cortical areas depending on species (Rakic, 1977; Van Essen, 1985). In the cat these distinct regions are called areas 17, 18, 19, 20, 21a, 21b and the lateral suprasylvian (LS) areas. The LS area is further subdivided. There are 3 visual areas in the rodent, termed 17,

18a and 18b (Caviness, 1975; Van Essen, 1985). Area 17, known as the striate visual cortex, is the target for ingrowing geniculate axons in the rodent (Simmons et al., 1982).

Each cortical area may be specialised to perform particular tasks. This is particularly clear in higher species. For instance, area V4 of the monkey visual cortex is responsible for colour vision, area V5 is responsible for motion analysis. Such functional specialisation is less clearly defined in cat and rodent.

The visual cortex of the mouse can be identified from other cortical regions when cut in the parasagittal plane, as it is the narrowest area of cerebral cortex (Caviness, 1975). The infragranular layers, 5, and 6, are narrower than in other cortical regions. Area 17 can be distinguished from both area 18a and 18b as it is slightly wider, with layer 4 having a higher density of cells. This is almost certainly to accommodate the ingrowing geniculate axons which synapse with cells in layer 4. Areas 18a and 18b are difficult to distinguish from each other based on cellular content, and can only be separated based on fibre content, with area 18a having a larger density of radial and tangential fibres (Caviness, 1975).

The inputs to the visual cortex of the adult cat and monkey are segregated, forming columns dominated by input from alternate eyes. That is, if one column is dominated by input from the left eye, its neighbour will receive input from the right eye (Wiesel and Hubel, 1963, 1965; Hubel and Wiesel, 1965; Rakic, 1977; Hubel et al., 1977; Levay et al., 1978; Shatz and Stryker, 1978). These columns are termed ocular dominance columns.

1.3.4 Summary of the anatomy of the adult visual system

In summary, light entering the eye penetrates the entire thickness of the retina and is then processed by the photorecepters. The resultant information is

transferred via the ganglion cells to the lateral geniculate nucleus (LGN) in the thalamus, and then to layer 4 of the visual cortex.

1.4 *DEVELOPMENT OF THE CEREBRAL CORTEX*

The Boulder Committee, (1970), identified and named five distinct regions of the developing cortex: from the ventricle to the pia, these regions are the ventricular zone, the subventricular zone, the intermediate zone, the cortical plate and the marginal zone. The cells of the cerebral cortex are generated from a population of stem cells, at the ventricular zone. After cell division, the newly formed cells migrate along the long processes of the radial glial cells to the cerebral cortex (Rakic, 1988; Hatten, 1990). These cells migrate in the order they were born, through the intermediate zone, which later becomes the white matter. The cortical plate, an intermediate structure, is the precursor of the cerebral cortex. The first cells to be produced by the stem cells arrive at the cortical plate, forming the deepest layers of this structure, and eventually the deepest layers of the cortex (Angevine and Sidman, 1961; Berry and Eayrs 1963).

The cortical cells migrate through the entire depth of the cortical plate until they reach the lower border of the marginal zone. Here they stop. Cells produced later in development migrate through and settle above the earlier generated cells, forming progressively more superficial layers. This process of cell division and migration results in the inside-out formation of the cerebral cortex (Berry et al., 1964; Fujita, 1964; Berry and Rogers, 1965; Fujita, 1966; Hicks and D'Amato 1968).

Studies in the rat using tritiated thymidine have suggested that neuronal cell division occurs at the ventricular surface (Berry and Rogers, 1965). It was shown that the synthesis of DNA, which occurs immediately before cell

division, takes place only at the ventricular zone, in the period E16-22. The stem cells divide, resulting in the generation of two daughter cells. One daughter cell migrates to take up its position within the developing cortical plate; this cell loses the ability to synthesise DNA and thus divide. The other daughter cell, however, remains in the ventricular zone and retains its ability to synthesise DNA and divide. This second daughter cell becomes responsible for the generation of subsequent cells, and thus subsequent layers of the cortex.

Cells of murine cortical plate are born between E13-17, with cells of layer 4 born on E14-15 (Caviness, 1982).

1.4.1 Development of a transient layer of cells: the subplate

Before the cortical plate is generated a population of transient cells is born. These cells migrate through the intermediate zone. Once above this zone, the early generated cells split, with half forming the marginal zone or future layer 1, while the other half remain deep, below the cortical plate. This layer of cells form a transient zone which has been shown to disappear shortly after birth. This zone is termed the subplate zone. The subplate zone is present in cats (Luskin and Shatz, 1985) and other species (human: Kostovic and Molliver, 1974; monkey: Rakic, 1974; rat: Bayer and Altman, 1990).

Wood, Martin and Price, (1992) showed that the subplate is present in the mouse. Using bromodeoxyuridine (BrdU) to birth date cells (Miller et al., 1988; Gillies et al., 1989), they have shown that a transient population of cells born on embryonic day 12-13 (E12-13) is situated immediately below the cortical plate. These cells probably die between birth and postnatal day 21 (P21), at which age the cortex is considered to have reached maturity (that is, the cortex is anatomically similar to that of the adult).

1.4.2 The role of the subplate

The role of the subplate in the cat was investigated by McConnell et al. (1989). They observed that the subplate neurons lay down one of the first cortical pathways. In this study, McConnell et al. (1989) raise the possibility that the early pathway formed by the subplate cells is necessary for the establishment of later permanent projections between the cortex and its many targets (see section 1.5.3 for more detail). Other similar studies have been carried out by Wahle et al. (1987), Valverde and Facal-Valverde (1988), Friauf et al. (1989 and 1990). Ghosh et al. (1990) highlighted a second role of subplate cells in the innervation of the correct cortical area by thalamic axons (see section 1.5.4 for more detail).

1.4.3 The development of ocular dominance columns

In the adult visual cortex inputs are arranged in a columnar fashion, the ocular dominance columns. Early in development these columns of inputs (described in more detail in section 1.3.3) from the two eyes overlap. As the cortex matures, the inputs gradually become segregated until they are distinct from each other.

1.5 *DEVELOPMENT OF THE GENICULOCORTICAL PATHWAY*

The LGN is situated in the thalamus. In the rat, cells of the LGN are born between E12-14 (Lund and Mustari, 1977). Fibres of the LGN project to the visual cortex and synapse with cells of layer 4.

1.5.1 Geniculocortical pathway formation and evidence for a waiting period

Rakic (1976) studied the development of the geniculocortical pathway in the monkey, using an autoradiographic method of orthograde axoplasmic and

transneuronal transport. He found that at E78 fibres have left the LGN and are on their way to the occipital cortex via the optic radiation. At E124, geniculate fibres have reached and are present below the cortical plate. The majority of fibres, however, have not invaded the cortex. At this stage, all cortical neurons are born and have completed their migration into the cortex. It is not until E144 that fibres invade the cortex. This study provides evidence suggesting that the growing geniculate axons undergo a period of waiting before they invade the cortex.

Studies carried out by Shatz and Luskin (1986) and Ghosh and Shatz (1992) confirm the existence of a waiting period in the development of the geniculocortical pathway of the cat. Geniculate axons grow to the cortex via the optic radiation, and then wait in the subplate zone before invading the cortical plate.

Ghosh and Shatz (1992), using the carbocyanine dye, 1,1'dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI), traced the progress of geniculate fibres in the cat and showed that LGN axons first arrive in the visual subplate at E36 (gestation period of the cat is 65 days). These fibres, however, are not detected in cortical layer 6 until E50. While waiting in the subplate, LGN axons extend terminal branches: it is likely that there are dynamic cellular interactions within the subplate at this time. Geniculate axons may not therefore be simply waiting during this period of development.

Wise and Jones in their 1978 study suggested the presence of a waiting period in the development of the pathway between the thalamus and the somatosensory cortex in rat (albino rat, Wistar strain). In this study autoradiographic, axonal degradation and horseradish peroxidase fibre tracing methods were used. They observed that at the time of birth a small number of fibres have invaded the somatosensory cortex and accumulated in the upper part

of layer 6. However, there was a heavy concentration of labelled fibres in the white matter below the cortex. This suggested that most of the fibres had not yet entered the cortex. Wise and Jones (1978) suggested, therefore, that the thalamic fibres undergo a stage of waiting before they invade the cortex.

1.5.2 Evidence disputing the waiting period

Lund and Mustari (1977) investigated the development of the geniculocortical pathway in the rat, using axonal degeneration techniques. They showed that geniculate axons begin to invade the telencephalon on E16. These fibres course through the optic radiation to the intermediate zone and reach the developing cortical plate on E18. They stated that a small number of axons invade the cortical plate at this time: the process of axon invasion is ongoing with more axons invading on following days. However, axon invasion is not complete until the day of birth, 4 days later. The definition of this period is difficult; Lund and Mustari labelled it a "waiting period". In the light of recent disputes over the presence or not of a waiting period in the rodent, however, I would challenge Lund and Mustari's use of this term and I would say rather, that they observed an "invasion period".

A similar investigation by Catalano et al. (1991) studied the invasion of axons into the somatosensory cortex, using the same strain of rat as Lund and Mustari, the Sprague-Dawley rat. This study disputed the existence of a waiting period. Using DiI to trace the progress of thalamocortical fibres, Catalano et al. (1991) showed that thalamocortical afferents reached the cortex on E16. At this time fibres were observed to be growing immediately under the cortical plate in the intermediate zone. On E17 thalamocortical fibres had begun their invasion into the cortical plate reaching the bottom of layer 6. At E18 more fibres had invaded, and reached the top of layer 6. By E20 individual growth cones can be

seen at the top of layer 6, and at the border of the differentiating layer 5 cells. By the day of birth dense ingrowth is observed in layers 6 and 5.

Both the study by Lund and Mustari (1977) and that of Catalano et al. (1991) suggest that there is no waiting period in the development of thalamocortical projections of the rat.

These findings have been supported by De Carlos and O'Leary (1992). Again using DiI to study the course of thalamocortical fibres in the rat, it was observed that thalamic axons begin their invasion of the cortical plate as early as E16.

The contradiction between these studies and that of Wise and Jones (1978) may lie with the experimental techniques used. The sensitivity of the autoradiographic method used by Wise and Jones has been challenged not only by Catalano et al. (1991) but also by Mason et al. (1990) in the study of the development of cerebellar climbing fibres in mouse.

The presence or absence of a waiting period in different species may reflect their different time-courses of development. The development of the cat and the monkey is spread over a long period of time. The growth of fibres from the thalamus to the cortex is quick in comparison to the generation, migration and differentiation of cortical cells. The thalamic fibres may wait for the cortex to catch up. The brain of the rodent, however, has a shorter period in which to develop, and so there may be no necessity for a waiting period.

1.5.3 Pathway pioneering by geniculate axons

It has been shown in both cats (McConnell et al., 1989) and rats (DeCarlos and O'Leary, 1992) that subplate cells send axons through the intermediate zone towards the thalamus, and that subplate neurons are the first cortical cells to produce outgrowth. These events occur at the same time as the

onset of outgrowth from the thalamus. In the rat, both sets of axons arrive in the internal capsule at the same time, making it possible that the axons come into contact with each other. However, DeCarlos and O'Leary (1992) conclude, on the basis of their experimental evidence, that these two axon populations have distinct pathways in the cortex making it unlikely that thalamic axons find their target by fasciculating with subplate axons. Alternatively, Molnar and Blakemore (1990) suggest that thalamic axons fasciculate with subplate axons using them for guidance to the cortex. It remains the case that the mechanism used by thalamic axons to reach the correct region of cortex is far from clear.

1.5.4 Innervation of correct cortical areas

In the reeler mutant mouse the neurons of the cortex are in abnormal positions resulting in the disruption of the normal layered structure. Despite the cells being in abnormal positions they still receive connections from their normal targets. That is, although layer 4 cells are in abnormal positions they will still receive connections from the LGN. These results indicate that the formation of connections with cortical neurons does not depend on the position of the cells (Caviness, 1976 and 1977; Steindler and Colwell, 1976; Stanfield and Cowan, 1979a and b; Stanfield et al., 1979).

A recent study by Ghosh et al. (1990) in the cat highlights the role of the subplate during the innervation of visual cortex by geniculate axons. In this study, the subplate was ablated and the path of growing geniculate axons was then followed using DiI. After ablation of the subplate, geniculate axons failed to recognise their target area, even though layer 4 cells were present. This suggests that the presence of the subplate is critical for visual cortical innervation by geniculate axons.

Mechanisms other than subplate guidance can be proposed to explain why thalamic axons will innervate specific regions of cortex. Chemical factors may be involved in this process.

1.6 *THE ROLE OF CHEMICAL GROWTH FACTORS*

Chemical factors have been shown to play an important role in the developing nervous system. Could it be that chemical factors are involved in the development of the geniculocortical pathway? There are two classes of chemical growth factor involved in development. Firstly, tropic factors mediate the influence one cell or tissue has on the direction of movement or outgrowth of another (e.g. Tessier-Lavigne et al., 1988; Heffner et al., 1990). Secondly, trophic factors mediate the ability of one tissue or cell to support another.

In other regions of the developing nervous system, diffusible trophic molecules have been shown to have important roles in promoting neuronal survival and growth (e.g. Lumsden and Davies, 1983 and 1986; Davies 1988). "Neurotrophic theory" (Purves, 1988) suggests that early changes in neuronal connectivity result from competition for a limited supply of trophic substances released by target tissues.

Cunningham et al. (1987) and Haun and Cunningham (1987) have suggested that a diffusible molecule released by the visual cortex has a role in the maintenance of the geniculocortical pathway. In their experiments, lesions of the occipital cortex cause degeneration of the LGN, but if blocks of agar saturated with medium conditioned by visual cortical explants are inserted into the lesions, many geniculate neurons are rescued.

1.6.1 Competition and plasticity in the developing brain

Chemical mechanisms have been shown to contribute to the selection of

specific pathways and targets by growing axons (e.g. Sperry, 1963; Stuermer, 1991). Competition between axons is also thought to be involved in this process. This is demonstrated by removing or manipulating the activity of one group of axons and observing the change in the projection pattern of the remaining axons. It is not clear what cells compete for, or what controls the different types of competition in different systems.

It is known that afferent cells often compete for trophic factors released by their target cells (Purves, 1988; Oppenheim, 1989). It has been proposed that some competitive interactions are controlled by the activation of NMDA glutamate receptors on target cells (Von der Malsburg and Singer, 1988; Constantine-Paton et al., 1990).

Plasticity is the term used to describe the ability of the developing or adult nervous system to undergo structural and functional changes. Domecini et el., (1991) studied the plasticity of the visual system in the rat. Monocular deprivation in rats during the critical period decreases the visual acuity of the deprived eye. In this study, the addition of NGF to monocularly deprived rats prevents the loss of visual acuity and sensitivity of the deprived eye. This suggests that NGF preserves the functional input to the cortex from the deprived eye. The mechanism of NGF action is, however, unknown.

1.7 *TERMINATION OF AXONS IN CORTICAL LAYER 4*

Yamamoto et al. (1989 and 1992), Molnar and Blakemore (1991) and Boltz et al. (1992) used a co-culture system to study the growth of geniculate axons into the visual cortex and their recognition of cortical layer 4. These studies provided anatomical and physiological evidence that the development of the geniculocortical pathway can be mimicked in vitro and they showed axons terminating in layer 4. The mechanism which controls this vital stage of

development is, however, unknown.

1.8 *SUMMARY OF THESIS*

1.8.1 Chapter 2: Development of cortical layer 4

In this chapter the migration of the cells which will form cortical layer 4, the target of geniculate axons, is investigated from shortly after their generation at the ventricular zone until they take up their final positions within the cortex. These cells are present in the cortical plate on E16, although they are not fully in place until around P3.

1.8.2 Chapter 3: Development of the LGN and the formation of the geniculocortical pathway

I investigated two related events in this chapter; firstly the birth of the LGN, using BrdU to birthdate cells; secondly, the timing of geniculate invasion into the murine visual cortex. The cells of the LGN are generated between E12-14 in the mouse, with the majority of cells born on E13 and E14. Using DiI, I was able to follow the geniculate axons as they traverse the optic radiation and innervate the visual cortex. This study showed that geniculocortical fibres invade the visual cortex before E17.

1.8.3 Chapter 4: *In vitro* study of geniculocortical development

The aim of this chapter was to search for the presence of a factor produced by the the occipital cortex which enhances outgrowth from LGN

explants. Using an organotypic co-culture system, I collected evidence which shows the existence of a factor produced by the occipital cortex.

1.8.4 Chapter 5: Investigation of the factor produced by the visual cortex

Here the factor produced by the visual cortex is further investigated. The following questions were addressed: (i) is this a diffusible factor, (ii) is it a chemotropic or a chemotrophic molecule and finally (iii) could this factor be nerve growth factor (NGF)? The factor was found to be freely diffusible in the culture medium. The evidence suggests that the factor is not required by embryonic LGN explants for survival. It is unclear whether or not it is having a tropic influence over the geniculate axons, although I consider it unlikely. NGF has no effect on outgrowth, making it unlikely that the factor produced by occipital cortex is NGF.

1.8.5 CHAPTER 6: SUMMARIES AND CONCLUSIONS

CHAPTER 2

DEVELOPMENT OF CORTICAL LAYER 4

2.1 *INTRODUCTION*

The cerebral cortex is a six-layered structure, with each layer composed of different types of cell (see section 1.3.3, The cerebral cortex: visual areas). The cortex is generated in an inside-to-outside manner, with cells of the deep cortical layers being born before those of the superficial layers (see section 1.4, Development of the cerebral cortex). Growing geniculate axons terminate in layer 4 of the visual cortex, forming synapses with the cells there. In this study, using an immunohistochemical technique similar to that of del Rio and Soriano (1989), Millar and Nowakowski (1988), Wood et al., (1991), I investigated the development of the cerebral cortex, particularly cortical layer 4.

As layer 4 is the target for ingrowing geniculate axons it was important to monitor the migration of cells destined for this layer into the cortex, to elucidate (i) when they first enter the cortical plate and (ii) when they take up their final positions. I needed to know these timings in the mouse so that I could choose appropriate days on which to culture the visual cortex (see Chapter 4: In vitro study of geniculocortical development). Most of the available literature concerned the development of rat visual cortex (Berry et al., 1964; Berry and Rogers, 1965; Hicks and D'Amato, 1968; Lund and Mustari, 1977), with only limited studies available on the mouse (Angevine and Sidman, 1961; Caviness, 1982). The data on rat cortical development, although useful, was not directly applicable to the mouse. For a start, there is a slight difference in the length of the gestation period, which is 22 days for the rat but only 21 days in the mouse. Also the times at which the various cortical layers are generated differ by 1-2 days between the two species.

Secondly, it was useful to compare the formation of layer 4 with that of the growing geniculate axons. I hoped that this study would help to answer

questions about whether the invasion of geniculate axons into the cortical plate coincides with (i) the first appearance of layer 4 cells there or (ii) the time at which these cells take up their final positions.

I labelled cells dividing on either E14 or E15 (the days on which layer 4 cells are born), allowing me to then follow their progresss on subsequent days. I decided to label with BrdU, as this is a relatively straightforward technique (discussed in 2.2.3; Immunohistochemistry, also section 2.4, Discussion), and has the advantage that the results of experiments are obtained almost immediately; tritiated thymidine experiments can take up to 8 weeks or so, to allow for autoradiographic exposure.

2.2 *MATERIALS AND METHODS*

2.2.1 Animals and BrdU injections

BALB/c mice from an isolated laboratory colony were mated overnight. On the following day (deemed to be embryonic day 1; E1) mice with a vaginal plug were removed from the colony. The pregnant mice were injected with a single dose (0.2mls of a 2mg/ml solution, i.p.) of BrdU dissolved in sterile saline, on either E14 or E15.

2.2.2 Preparation of tissue

Tissue was collected from embryonic, neonatal and adult animals (see Table 1 for more details).

Fetuses were removed from the mother after an overdose of sodium pentobarbitone (0.3mls of a 12mg/ml solution i.p.). The heads were placed in

4% paraformaldehyde in phosphate buffer for 24 hours. The brains were dissected out and placed in the same fixative for 1 hour, then in phosphate buffer for up to 24 hours. The tissue was then dehydrated in alcohol, cleared overnight with chloroform and embedded in wax. 20um parasagittal sections were cut using a microtome and mounted onto glass slides precoated with a 0.01% solution of poly-L-lysine. The tissue was dewaxed in xylene for 20 minutes.

Postnatal mice were killed with an overdose of sodium pentobarbitone (0.3mls at 12mg/ml for adults and 0.15mls at 12mg/ml for early postnatal animals). These animals were then perfused transcardially first with saline, immediately followed by 4% paraformaldehyde in phosphate buffer. The brains were then removed and post-fixed with a similar concentration of paraformaldehyde for up to 2 hours. The tissue was placed in phosphate buffered sucrose (with 20% sucrose) overnight to equilibrate. 30um parasagittal sections were cut using a freezing microtome. These sections were mounted onto glass slides as before.

2.2.3 Immunohistochemistry

Sections were treated with 1% trypsin in 1% $CaCl_2$ at 37^{o}C, to permeabilize the cell membranes; adult tissue was treated for 20 minutes, fetal and early postnatal tissue for 30 minutes. The sections were then washed with tris-buffered saline (TBS at pH 7.6) for 5 minutes before being treated with 1N HCl at 60^{o}C for 8 minutes, to denature the double-stranded DNA molecules and expose the incorporated BrdU molecules. Sections were washed with tap-water, then preblocked with normal rabbit serum (NRS) in TBS (1:4 NRS:TBS) for 10 minutes. The sections were incubated with anti-BrdU (5ul/ml) for 30 minutes and then washed twice in TBS for 5 minutes each. A second preblocking

treatment with NRS followed. The sections were next treated with a biotinylated secondary antibody (5ul/ml) for 30 minutes. When this incubation was complete, the sections were once again washed with TBS, then treated with avidin and biotinylated horseradish peroxidase (HRP) for 30 minutes. Following a final wash with TBS, the HRP was reacted with diaminobenzidine and hydrogen peroxide. The sections were counterstained with cresyl fast violet and differentiated in alcohol and water. Once dry, the sections were coverslipped with DPX mountant.

TABLE 1.

INJECTION OF BrdU	FIXATION DATE
E14	E16
E14	E17
E14	E18
E14	E19
E14	P0
E14	P3
E14	P7
E14	ADULT
E15	E16
E15	E17
E15	E19
E15	P0
E15	P4
E15	P7
E15	ADULT

Table 1 Summary of BrdU experiments detailing the date of BrdU injection and subsequent fixation. Several pups were processed, however due to the fragility of the tissue data was available from only one animal in each experimental group.

2.2.4 Analysis

The above immunohistochemical technique was used to identify a population of cells born on E14 and E15. BrdU labelled nuclei appear dark brown. The labelling of the cells varied with some being densely labelled: more than half of the nucleus was dark brown. Others were lightly labelled: less than half of the nucleus was dark brown. It is likely that heavily labelled cells were generated by stem cells at the ventricular zone on the day the injection was made. Many of the lightly labelled cells may be the product of a second round of cell division. That being the case, these cells should contain half of the amount of BrdU found in the first generation of cells, therefore producing lightly labelled cells. The intensity of label has been used previously to determine the number of divisions that the stem cell has undergone before the labelled cell was generated (Smart and Smart, 1982).

Figure 1 is a schematic diagram similar to the camera lucida drawings made of each section of cortex, detailing the method of analysis. For each brain, two camera lucida drawings were made from adjacent sections through the occipital cortex. A box 500um wide, with the depth divided into 50 (embryonic tissue) or 100um (postnatal and adult tissue) bins, was placed over the section and used to record the positions of labelled cells. With embryonic tissue, the box was divided into 50um bins and covered the entire section, from the pia to the ventricle. The cortical plate/intermediate zone (CP/IZ) border was used as a baseline to fit the bins. All bins above this line were assigned positive values, while those below it were allocated a negative value. However, with neonatal and adult tissue there was no need to analyse the entire section as all labelled cells had migrated into the cortex. In these cases, I divided each box into 100um-deep bins, using the white matter/cortex border as a zero baseline. The number of labelled cells in each bin was counted, and used to construct

histograms of the numbers of labelled cells against depth in the cortex. Using these histograms I was able to follow the progress of labelled cells as they migrated through the cortical plate to their final positions.

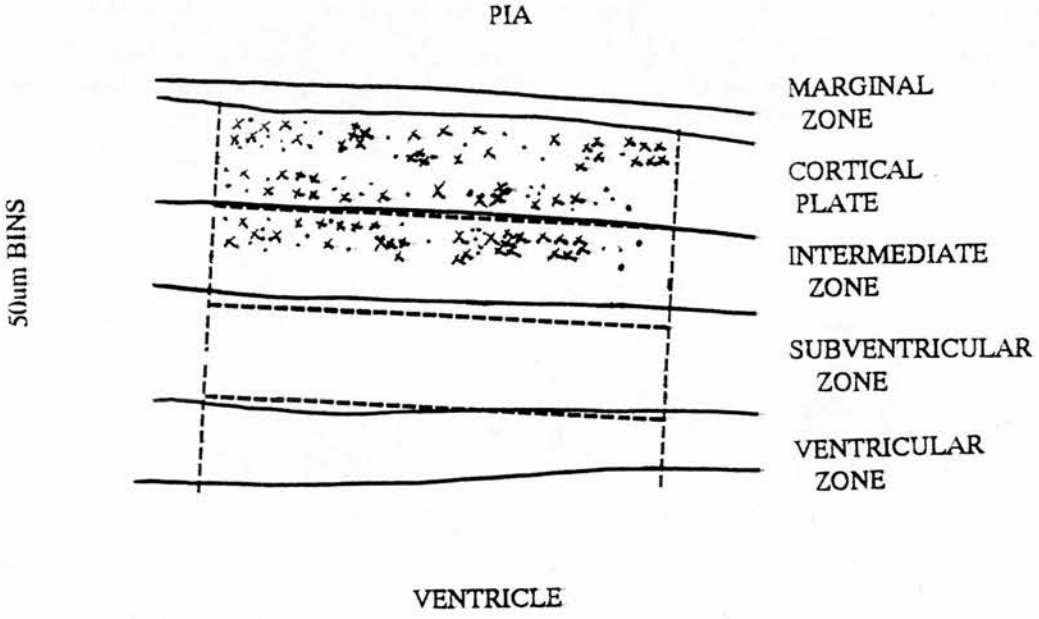


Fig. 1 Schematic diagram detailing the method of analysis of BrdU labelled sections. The labelled cells are indicated by x and o, with x being densely labelled cells and o lightly labelled cells. The five zones described by the Boulder Committee (1970) are identified; these are, from the pia to the ventricle, the marginal zone, the cortical plate, the intermediate zone, the subventricular zone and the ventricular zone. The broken lines surrounds the boxes used to count the numbers of labelled cells. The cortex has been segregated into 50um bins, with the bins above the cortical plate given positive values and those below it negative values (for example, -50um indicates the bin directly below the border of the cortical plate). Scale bar, 50um.

2.3 *RESULTS*

2.3.1 BrdU labelling

The BrdU label was confined to the cell nucleus. This label varied in intensity between cells (Fig. 2). The densely labelled cells were judged to have 50% or more of the nucleus labelled, while the lightly labelled cells had less than 50% of their nucleus labelled. All judgements were made by eye. Figure 2 is a photomicrograph of BrdU labelled cells in the visual cortex of a mouse on E18 after an injection of BrdU on E14, and shows both lightly and densely labelled cells.

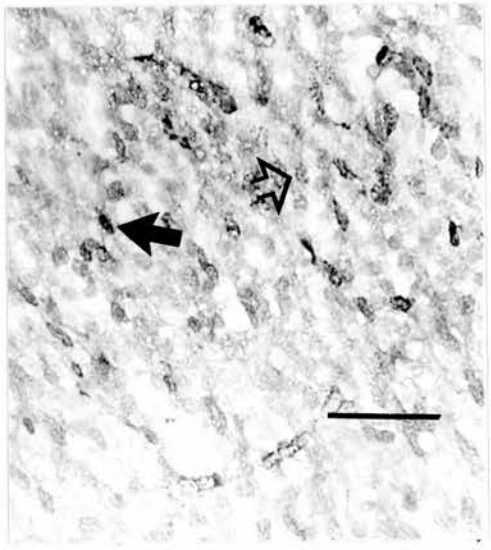
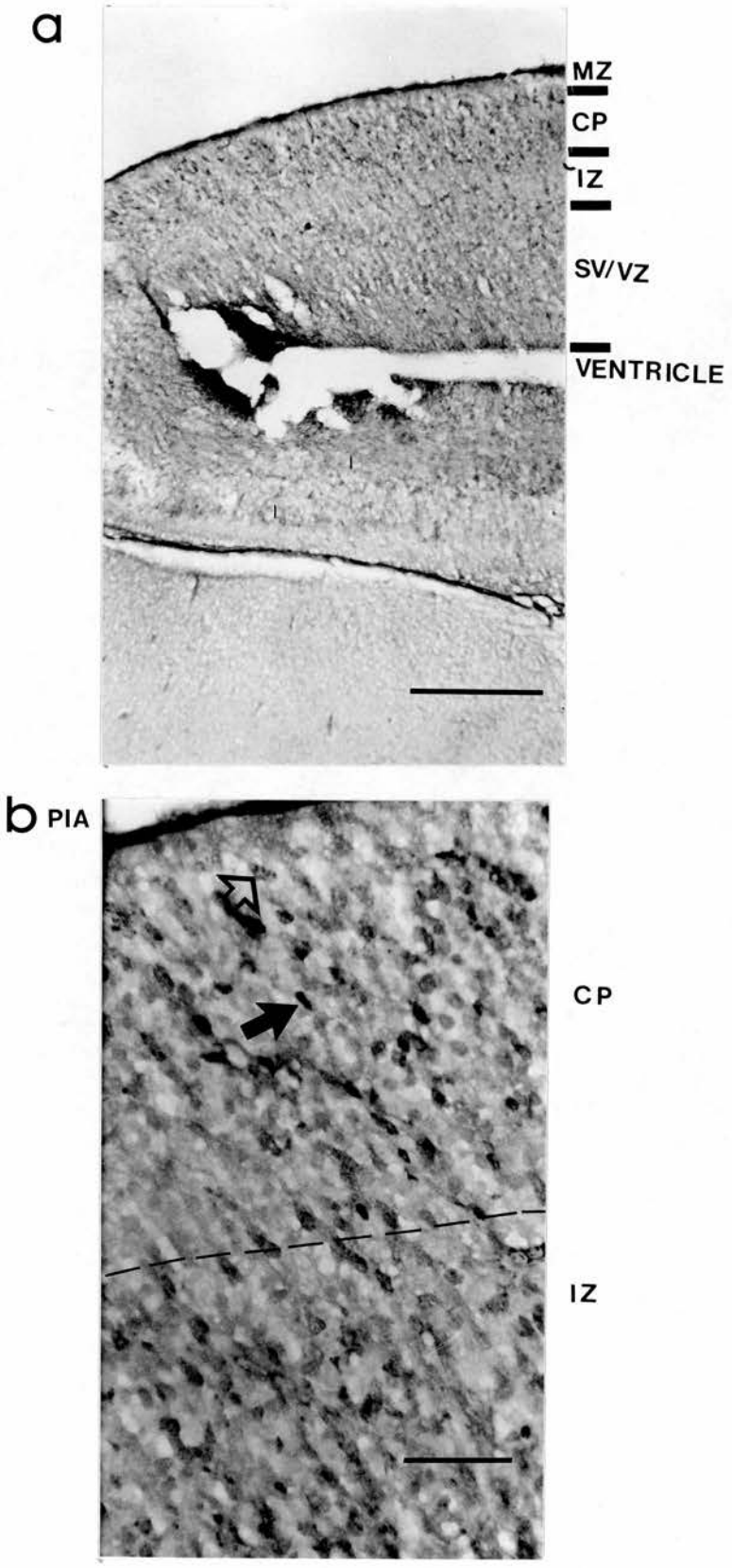


Fig. 2 Photomicrograph of a BrdU labelled section injected with BrdU on E14 and fixed on E18. Here a densely labelled cell is highlighted by a filled arrow, with a lightly labelled cell indicated by an open arrow. Scale bar, 50um.

2.3.2 Position of labelled cells

The position of labelled cells was investigated (Fig. 3). Fig. 3a is a photomicrograph of a BrdU labelled section of visual cortex; the injection of BrdU was made on E15, and the tissue was fixed on E17. The five zones identified by the Boulder Committee (1970), that is, the marginal zone, the cortical plate, the intermediate zone, the subventricular zone and the ventricular zone, are identified. It is evident from Fig. 3a that labelled cells are already present in the cortical plate at E17, although a large number are still present in the intermediate zone. Fig.3b shows more clearly that labelled cells are present in the cortical plate; the pial surface of the brain is at the top left. Some of the labelled cells are located just below the cortical plate.

Fig. 3 Photomicrographs of a section of visual cortex labelled with BrdU on E15 and fixed on E17. (a) A low power photomicrograph showing the 5 zones identified by the Boulder Committee (1970). (b) The same section at higher power; the pial surface is present (the black line in the top left corner). Both densely and lightly labelled cells are highlighted: densely labelled cells by a filled arrow and lightly labelled cells by an open arrow. The broken line indicates the border between the cortical plate and the intermediate zone, as identified with nissel counterstain. Abbreviations: MZ, marginal zone; CP, cortical plate; IZ, intermediate zone; SV/VZ, subventricular/ventricular zone. Scale bars, a=150um, b=20um.

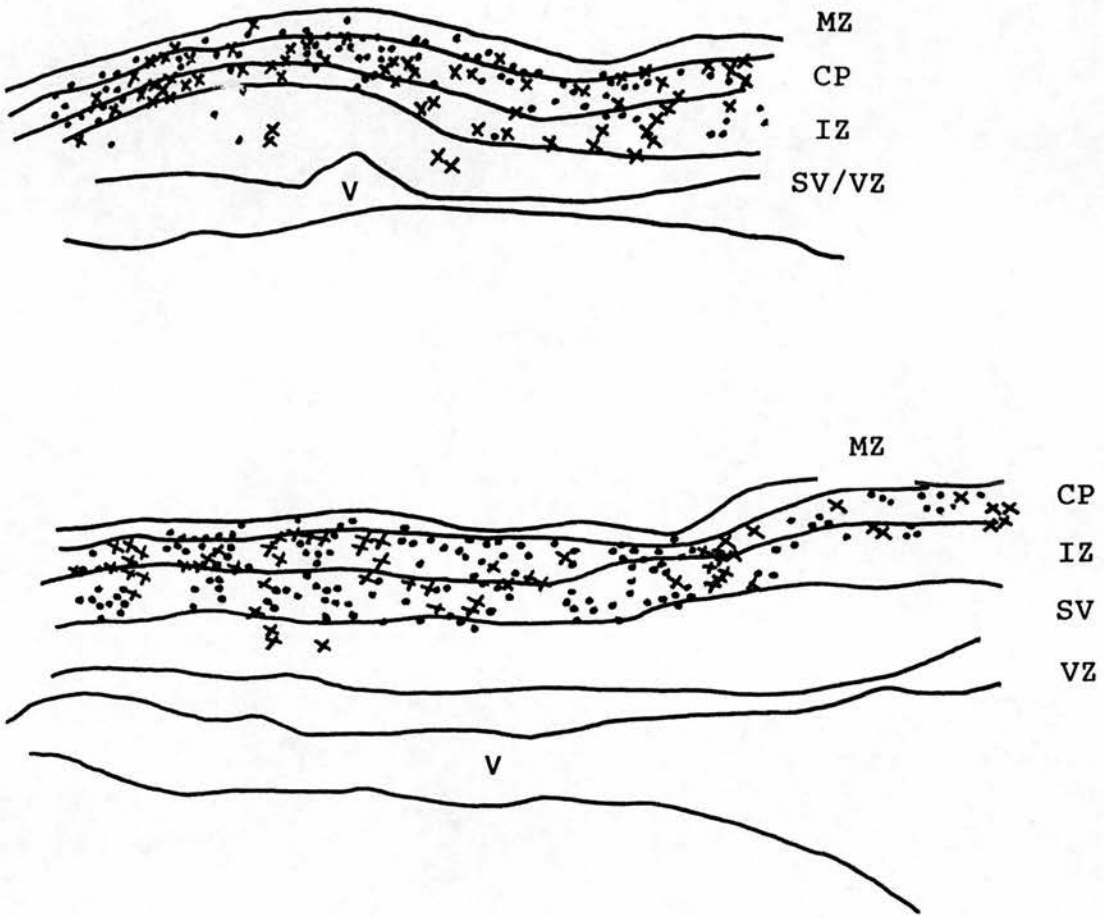

a
MZ
CP
IZ
SV/VZ
VENTRICLE
b PIA
CP
IZ

Camera lucida drawings were made of each section analysed. Figures 4 and 5 show examples of these drawings (Fig. 4 presents data collected from E16-19 animals injected with BrdU on E14, while Fig. 5 shows data collected from E16-19 animals injected with BrdU on E15). These drawings detail the migration of labelled cells from the ventricular zone into the cortical plate. Both Figures 4 and 5 show two important observations. Firstly, as the tissue gets older the cortical plate gets thicker. The cortical plate roughly doubles its depth between E16 and E19. I often observed slight variations between animals in the thickness of the telencephalon fixed at the same developmental stage. This may be caused by several factors. First, the degree of fixation may be important; that is, a well-fixed tissue will shrink quite considerably, so if the fixation is not very good the tissue will not shrink as much. Second, it is likely the there would be slight variations in the age of the tissue. The animals are mated over a 12 hour period and some females could be plugged at the beginning of the period while others could be plugged at the end. This would make some pups at most half a day older than others, and therefore slightly larger. Third, the size of the litter also plays a role in the size of the resultant pups. That is, pups from a large litter tend to be smaller than those from smaller litters. Finally, there will always be slight variations in the size of the fetus/pup due to other factors.

Fig. 4 Camera lucida drawings of sections of visual cortex labelled with BrdU on E14 and fixed on (a) E16, (b) E17, (c) E18, and (d) E19. Densely labelled cells are indicated by X, and lightly labelled cells o. The different zones identified by the Boulder Committee (1970) are indicated. Abbreviations as in Fig. 3. Scale bars represent, (a) and (b) 150um, (c) and (d) 100um.

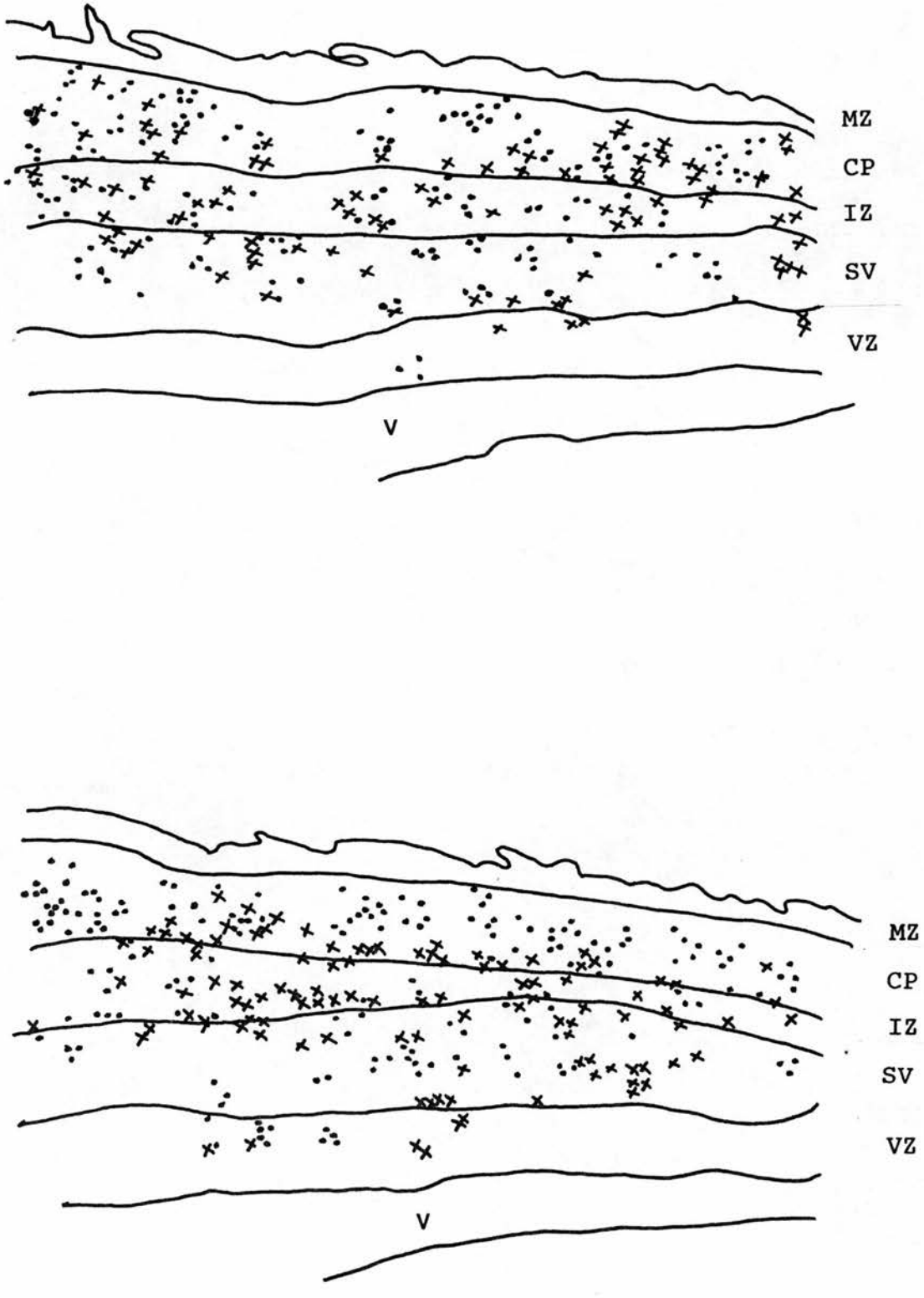
E14 BrdU INJECTION
E16 FIX

a



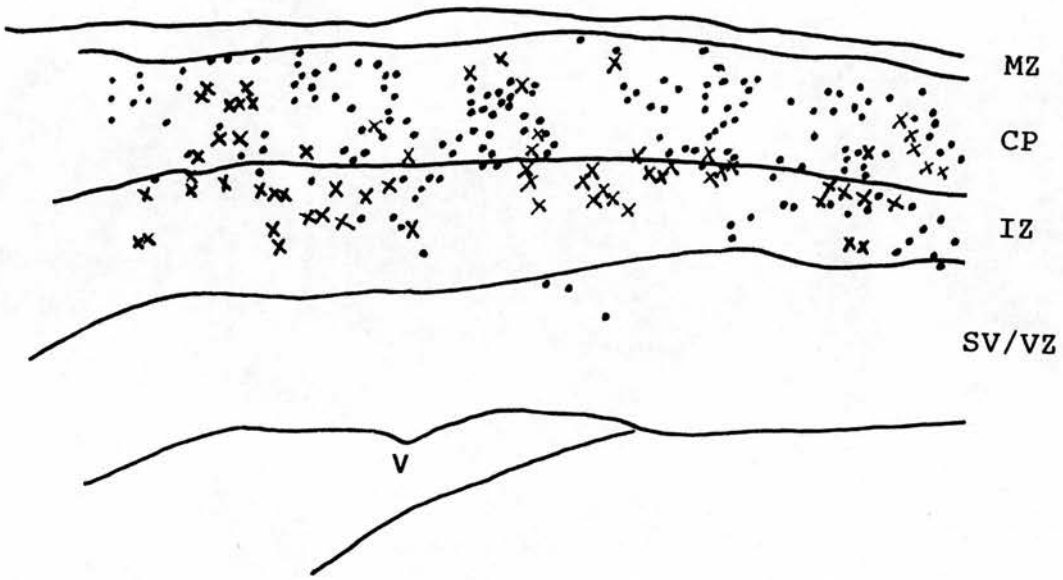
E14 BrdU INJECTION
E17 FIX

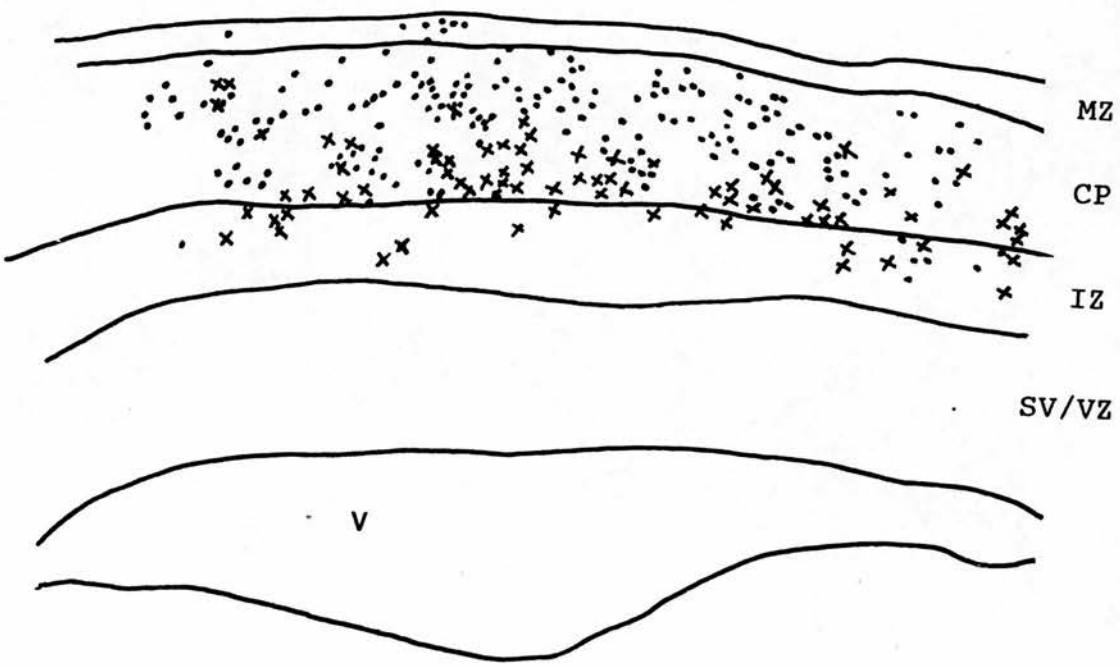
b



E14 BrdU INJECTION
E18 FIX

c





E14 BrdU INJECTION
E19 FIX

d

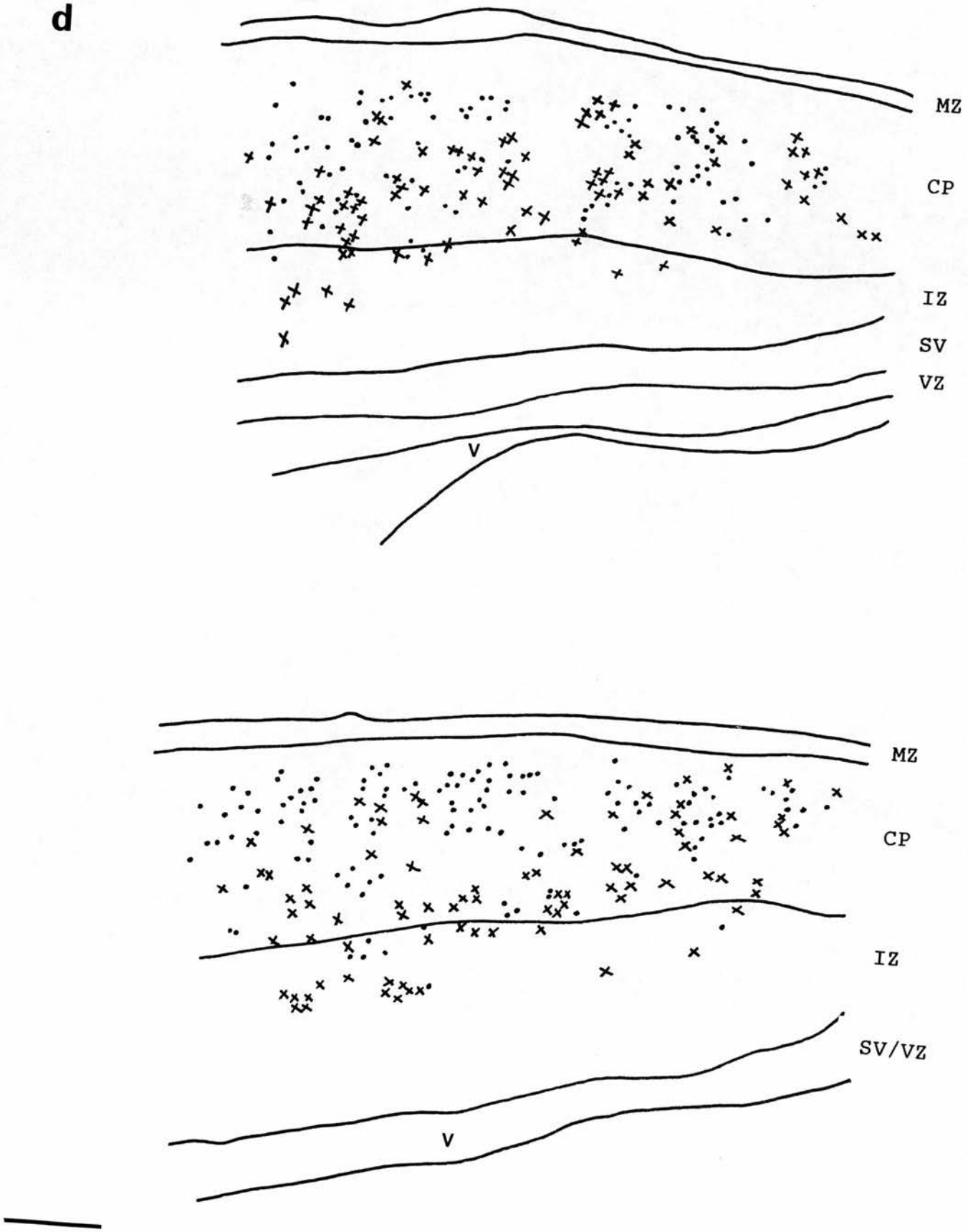
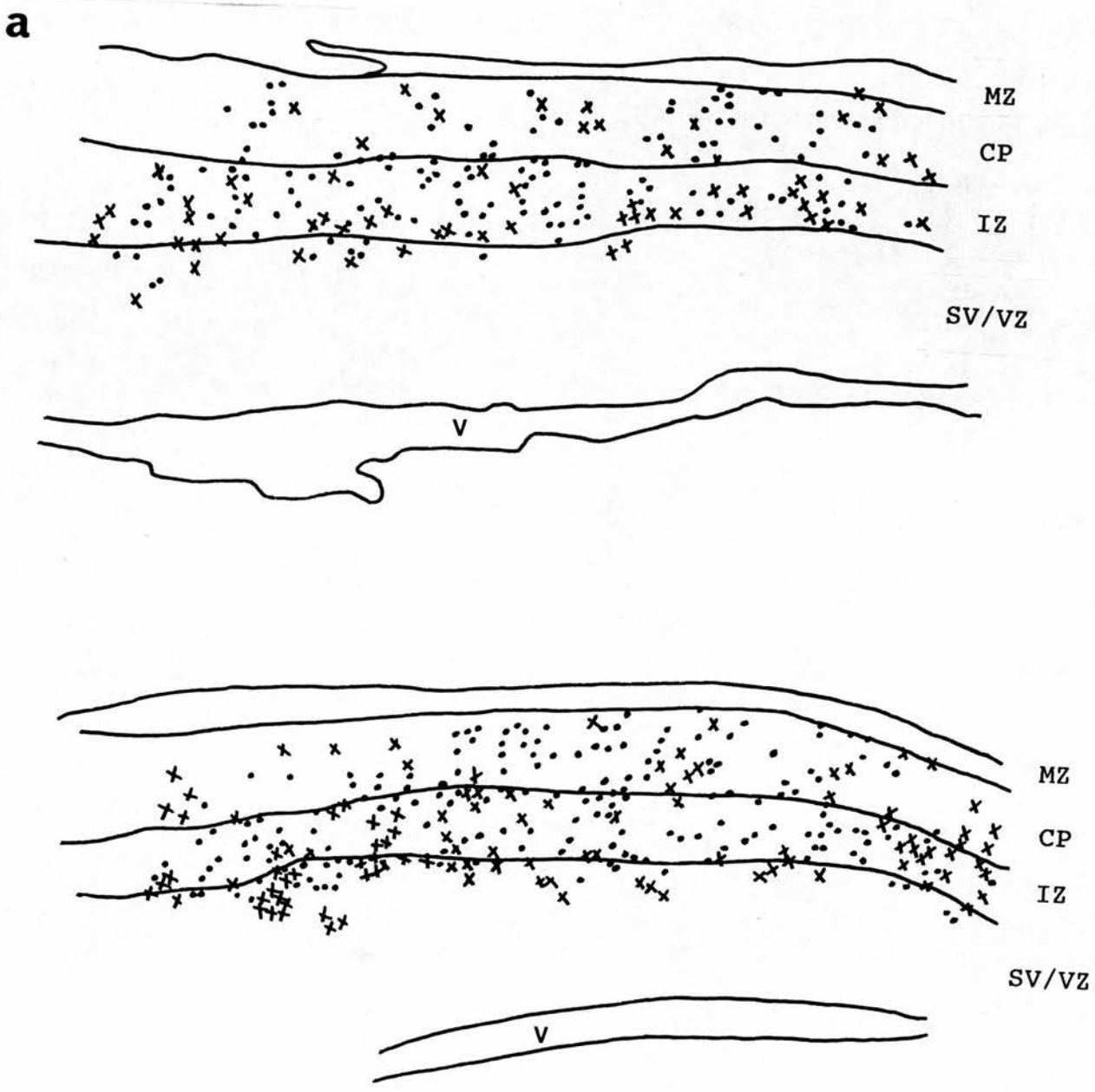


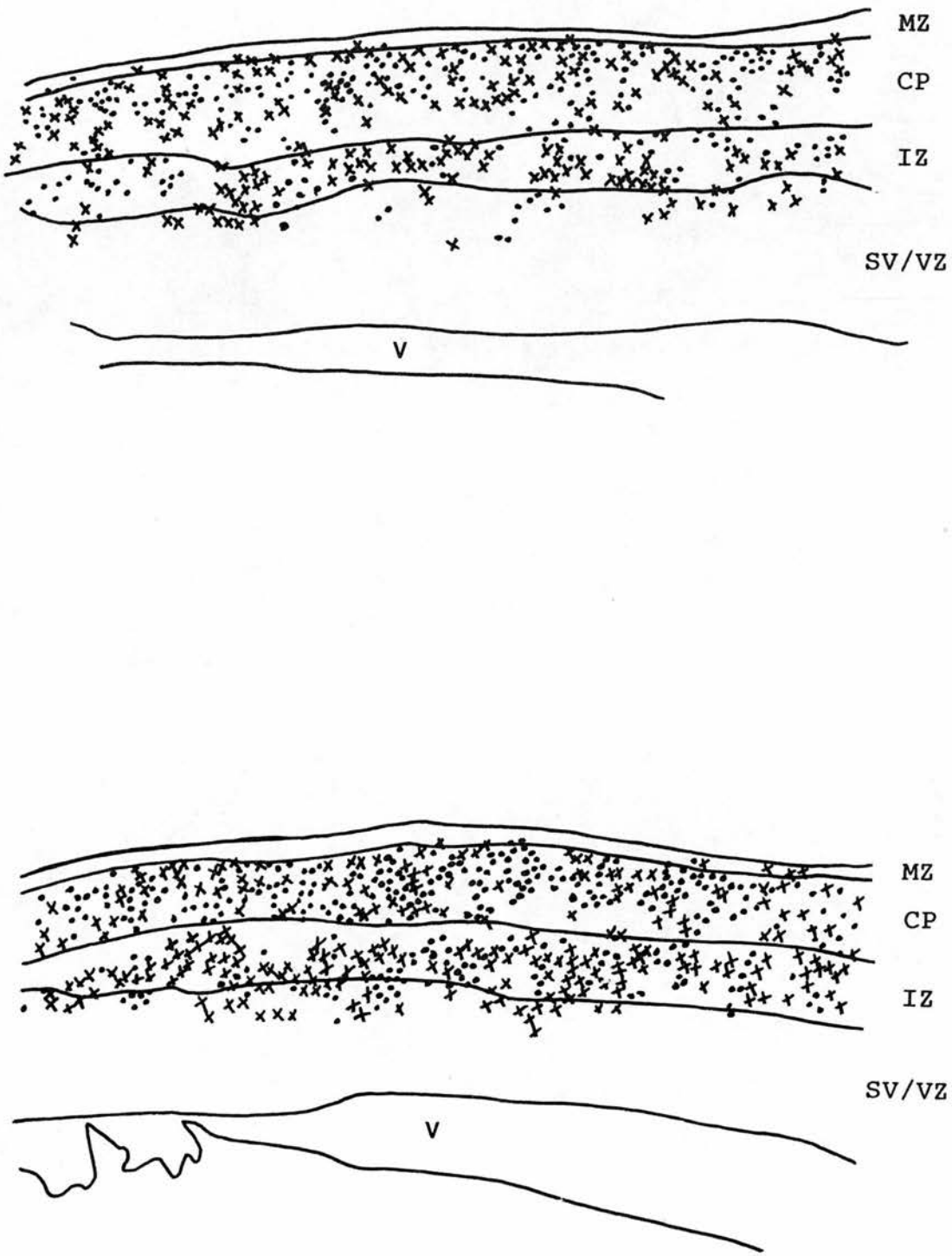
Fig. 5 Camera lucida drawings of sections of visual cortex labelled with BrdU on E15 and fixed on (a) E16, (b) E17 and (c) E19. Conventions are as in Fig. 4. Scale bars, 150um.

E15 BrdU INJECTION
E16 FIX



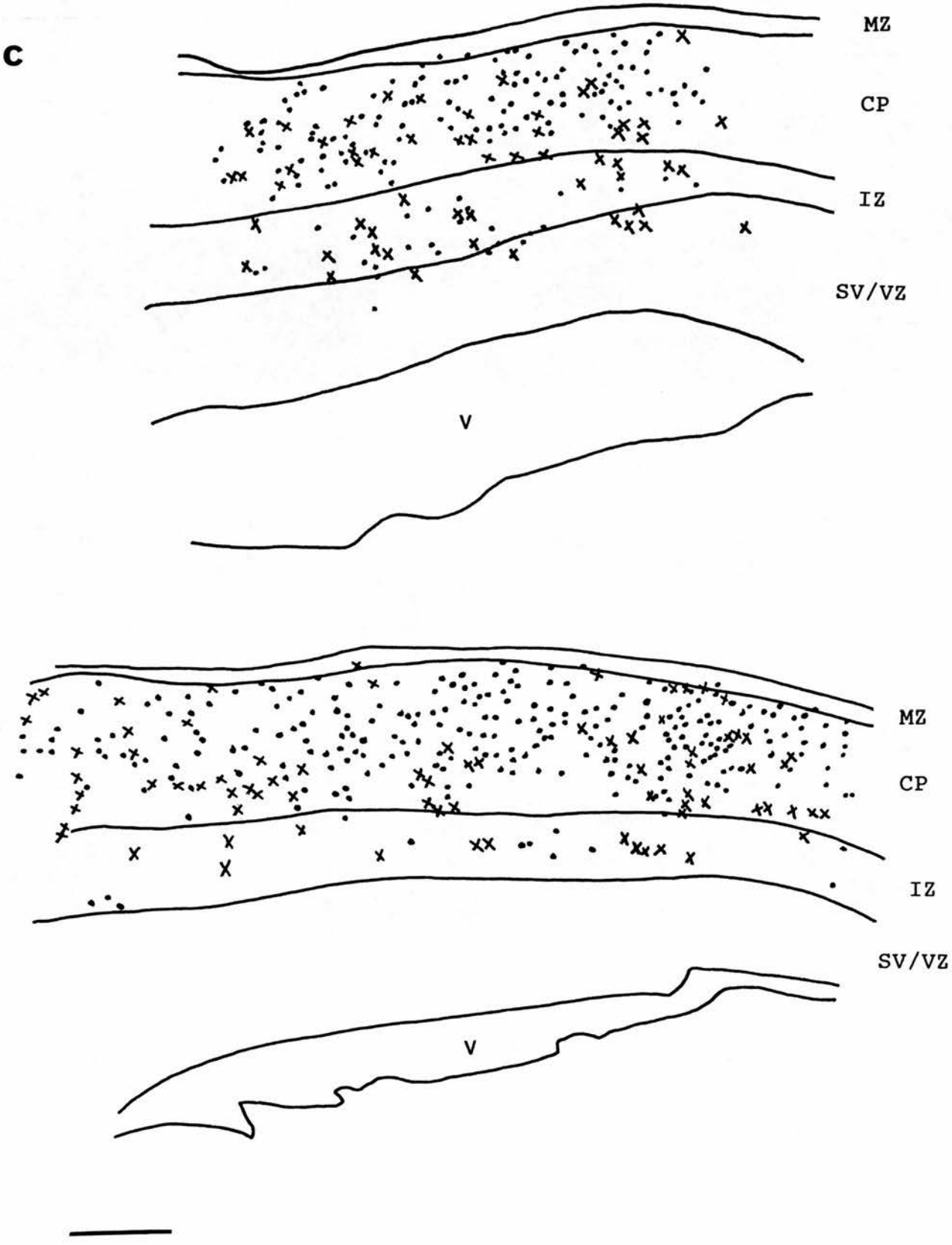
E15 BrdU INJECTION
E17 FIX

b



E15 BrdU INJECTION
E19 FIX

C

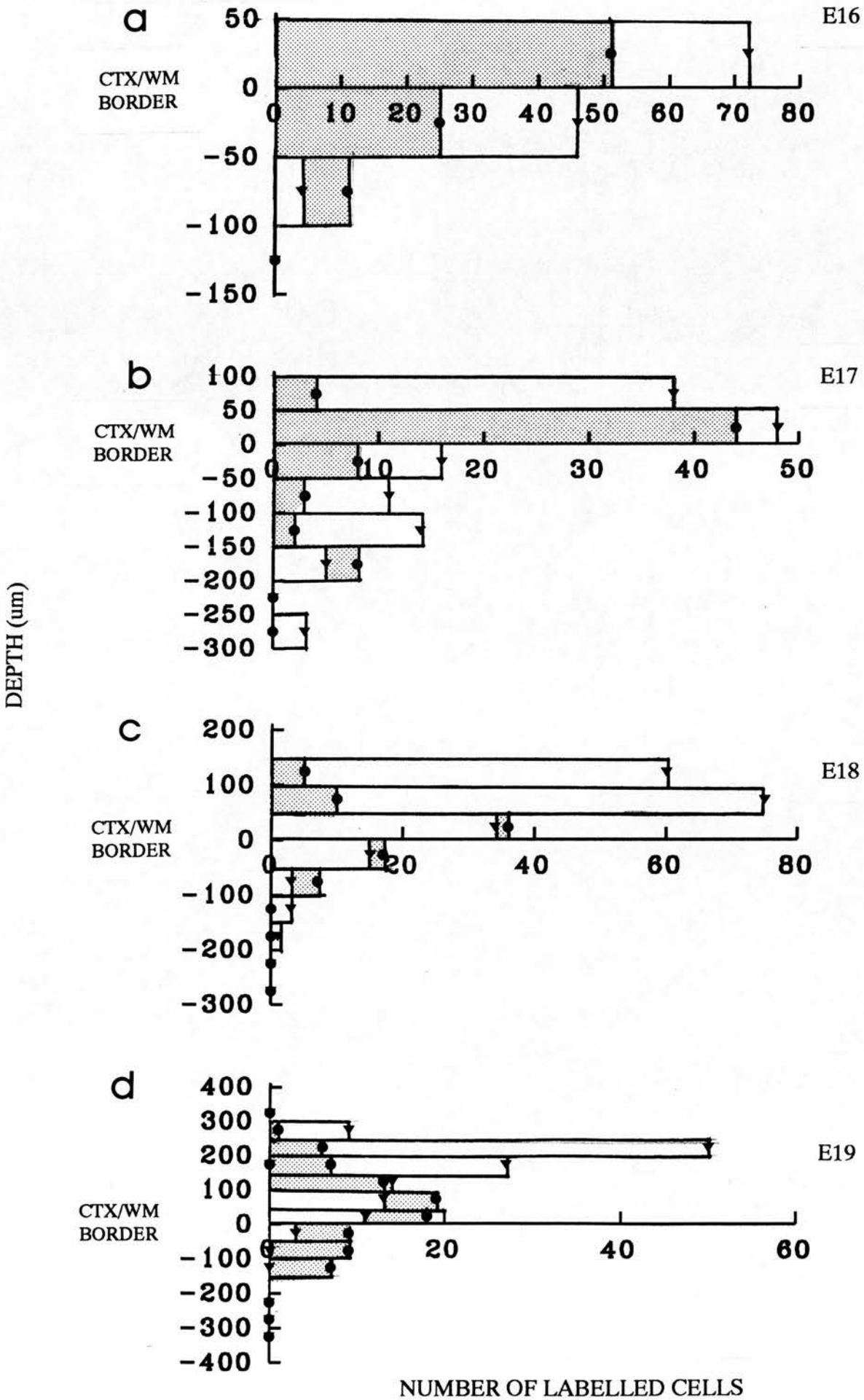


The second observation illustrated by these figures concerns the migration of the labelled cells. I first considered the migration patterns of cells labelled on E14 (Fig. 4). At E16 and E17 labelled cells can be seen throughout the telencephalon. However, a large proportion of the labelled cells have already invaded the cortical plate (Fig. 4a and b). By E18, most of the labelled cells have migrated into the cortical plate, with only a small number located in the upper half of the intermediate zone (Fig.4c). At E19, an even greater proportion of labelled cells are in the cortical plate (87%), with the remaining 13% of labelled cells largely confined to the upper half of the intermediate zone (Fig. 4d). At E19 a slight gap between the labelled cells and the top of the cortical plate has appeared. This gap is filled by non-labelled cells, indicating that a new population of cells has been generated and has migrated through the E14 labelled cells to take up more superficial positions.

The results presented in Fig. 5 confirm the observations of the E14 BrdU injections (Fig. 4). Labelled cells are present in the cortical plate on E16, despite being generated only one day previously (Fig. 5a). Further migration of cells into the cortical plate is observed on E17 (Fig. 5b). By E19 the majority of cells are present in the cortical plate (Fig 5c). Unlike the E14 labelled cells however, cells are located throughout the cortical plate and there was no gap between the labelled cells and the top of the cortical plate. The difference between the positions of E14 and E15 labelled cells on E19 (compare Figs. 4d and 5c), is explained by the inside-to-outside formation of the cerebral cortex.

The histograms in Figs. 6 and 7 support and extend the results presented above. All cells labelled on both E14 and 15 are present in the cortex at P0 (the day of birth); no labelled cells were found below the cortex/white matter border at this time.

Fig. 6 Histograms indicating the position of cells, labelled on E14, and the number of both lightly and densely labelled cells in each bin. The distributions of cells are after fixation on (a) E16 (b) E17, (c) E18, (d) E19, (e) P0, (f) P3, (g) P7 and (h) adult. The cortex/white matter (CTX/WM) border is represented by the x-axis. Positive values in the y-axis represent areas above the CTX/WM border, negative values represent all areas below the cortical plate. The bars representing densely and lightly labelled cells have been superimposed on top of each other, with whichever category has the least number of cells placed at the front. Filled bars represent the number of densely labelled cells and open bars lightly labelled cells.


E14 BrdU INJECTION
a
E16
CTX/WM BORDER
50
0
-50
-100
-150
0 10 20 30 40 50 60 70 80
b
E17
CTX/WM BORDER
100
50
0
-50
-100
-150
-200
-250
-300
0 10 20 30 40 50
c
E18
CTX/WM BORDER
200
100
0
-100
-200
-300
0 20 40 60 80
d
E19
CTX/WM BORDER
400
300
200
100
0
-100
-200
-300
-400
0 20 40 60
DEPTH (um)
NUMBER OF LABELLED CELLS

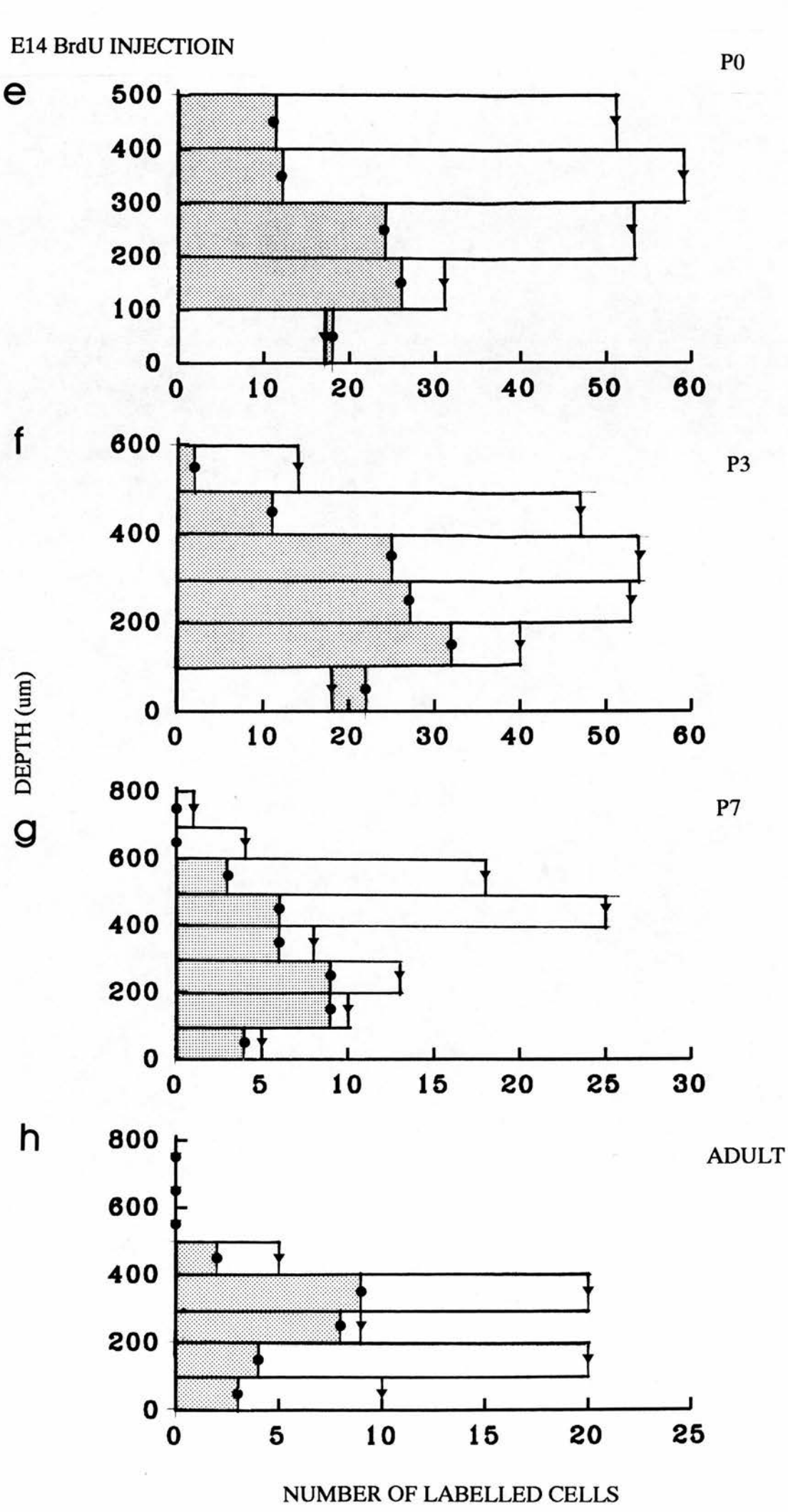
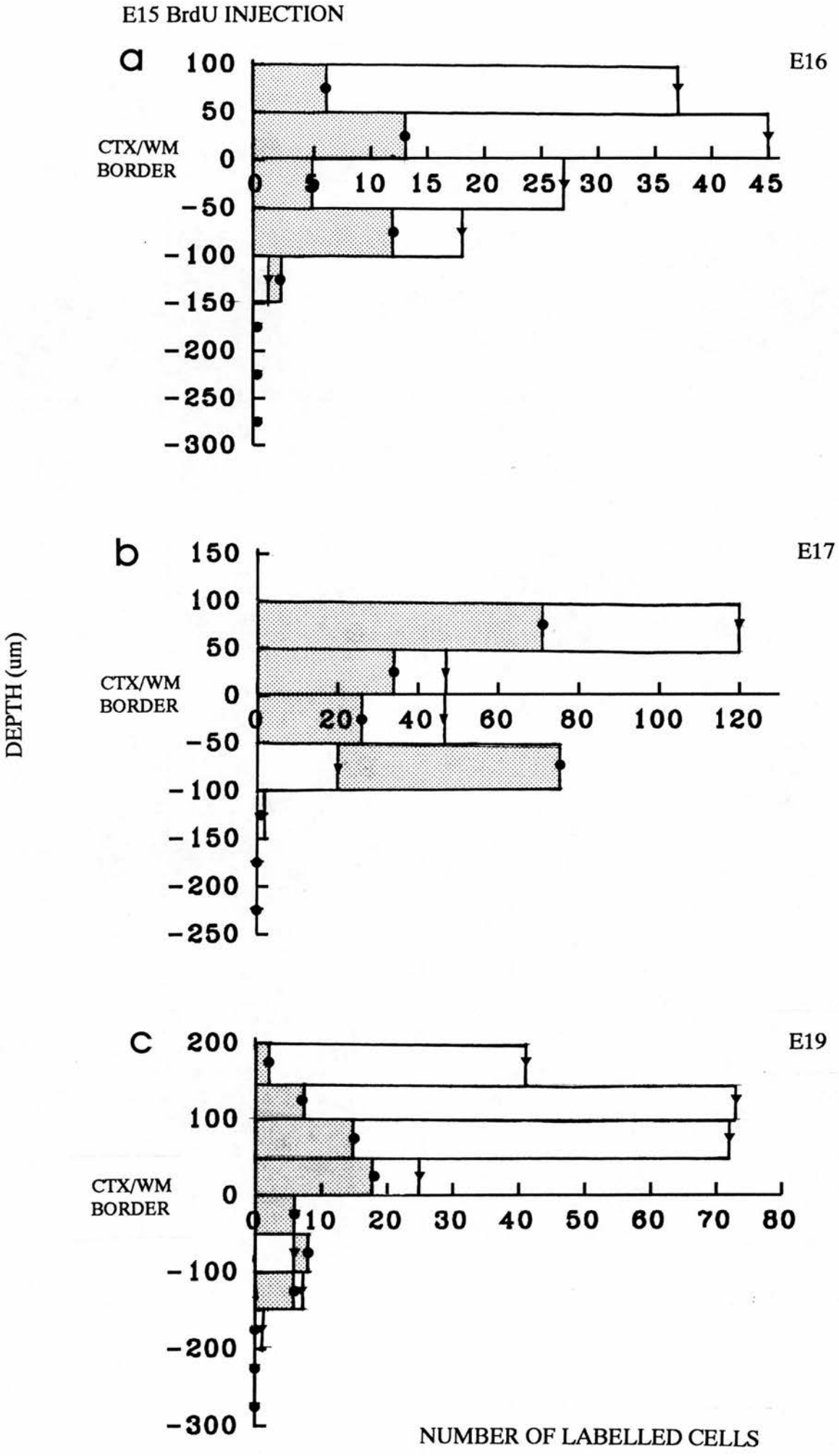

E14 BrdU INJECTIOIN
P0
e
500
400
300
200
100
0
0 10 20 30 40 50 60
f
P3
600
400
200
0
0 10 20 30 40 50 60
DEPTH (um)
g
P7
800
600
400
200
0
0 5 10 15 20 25 30
h
ADULT
800
600
400
200
0
0 5 10 15 20 25
NUMBER OF LABELLED CELLS

Fig. 7 Histograms detailing the positions and numbers of cells labelled with BrdU on E15. (a) Shows the distribution of cells after fixation on (a) E16, (b) E17, (c) E19, (d) P0, (e) P4, (f) P7 and (g) adult. The conventions are as in Fig 6.


E15 BrdU INJECTION
a
E16
CTX/WM BORDER
100
50
0
-50
-100
-150
-200
-250
-300
0 5 10 15 20 25 30 35 40 45
b
E17
CTX/WM BORDER
150
100
50
0
-50
-100
-150
-200
-250
0 20 40 60 80 100 120
DEPTH (um)
c
E19
CTX/WM BORDER
200
100
0
-100
-200
-300
0 10 20 30 40 50 60 70 80
NUMBER OF LABELLED CELLS

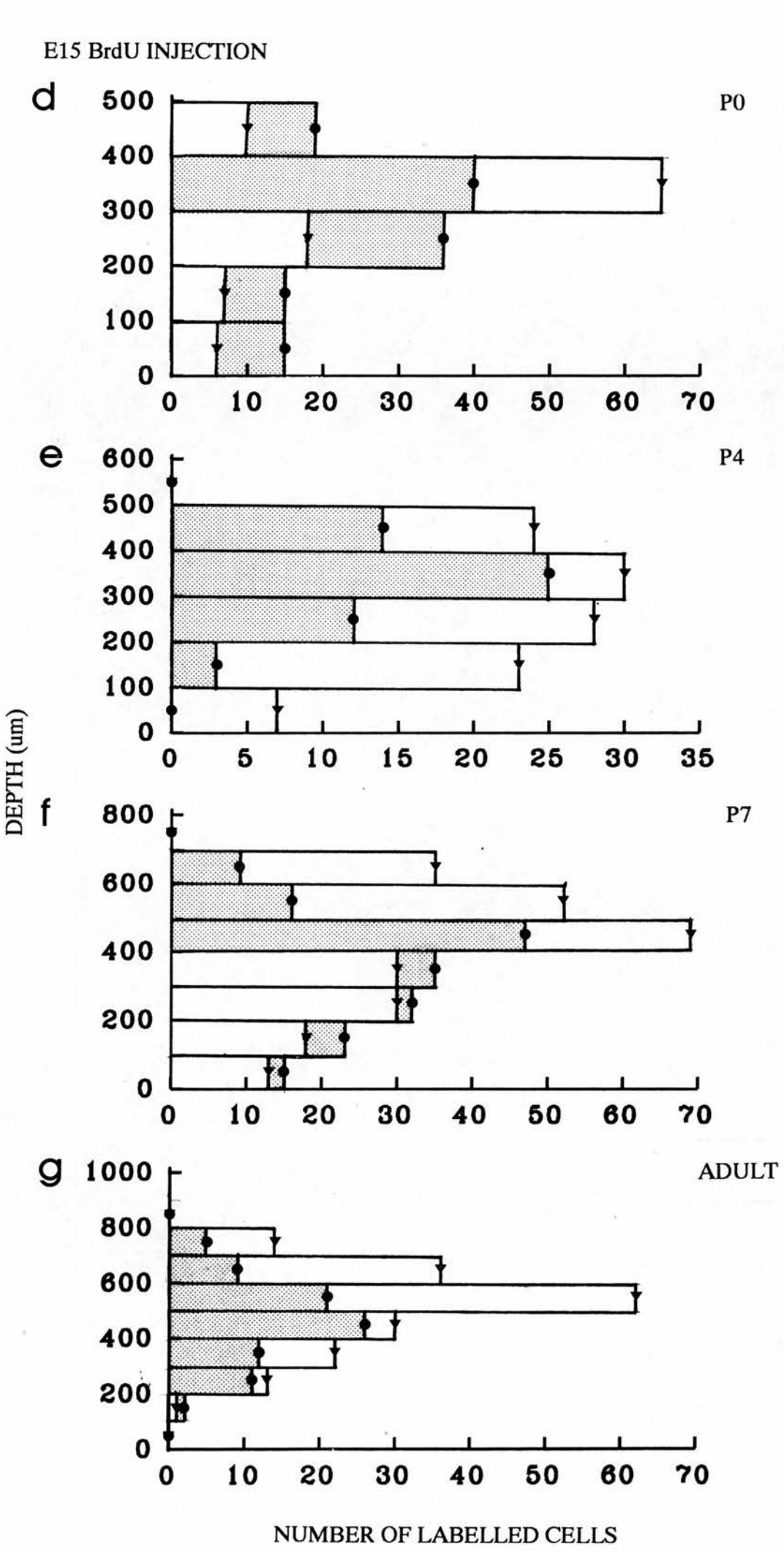
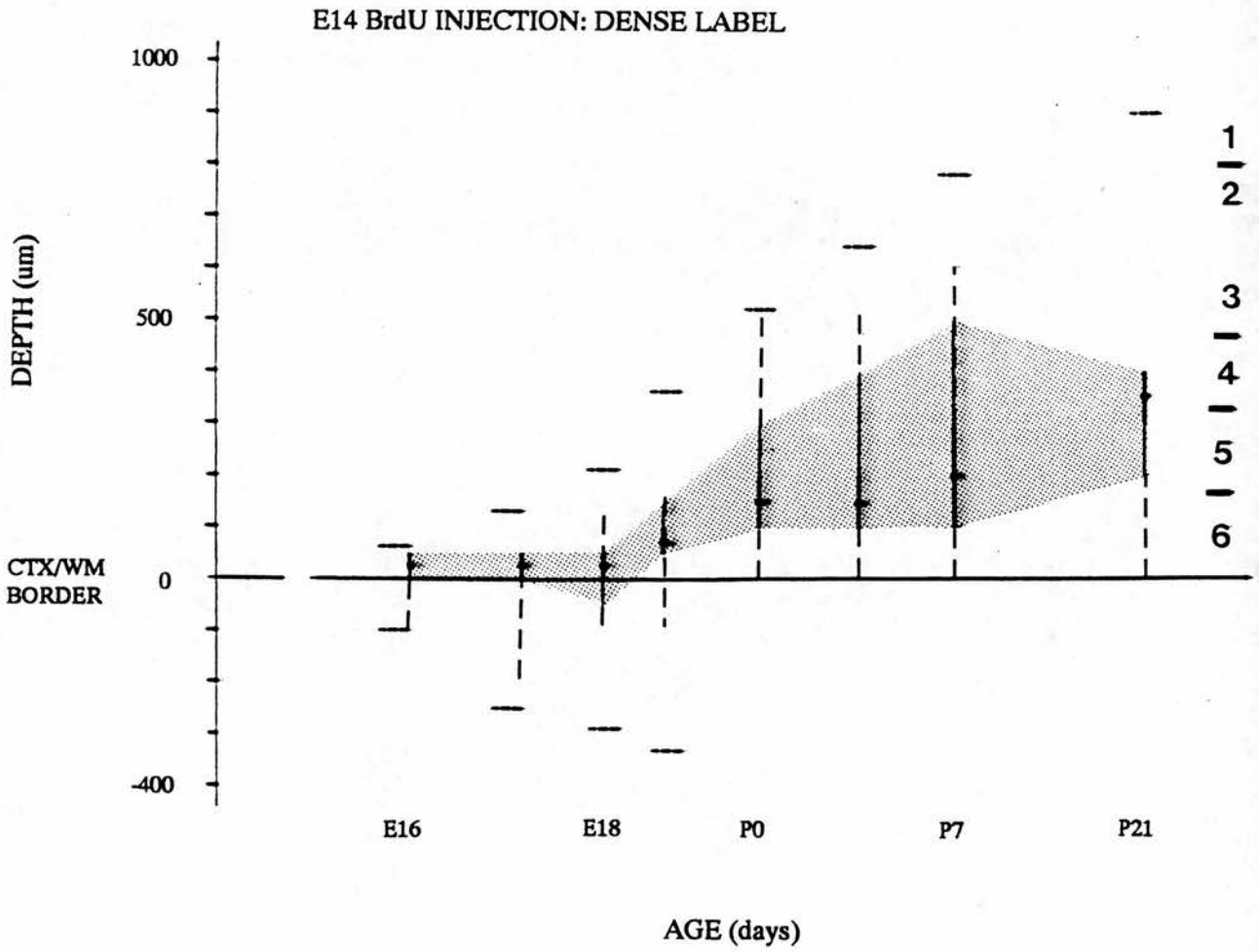

E15 BrdU INJECTION
d
P0
500
400
300
200
100
0
0 10 20 30 40 50 60 70
e
P4
600
500
400
300
200
100
0
0 5 10 15 20 25 30 35
DEPTH (um)
f
P7
800
600
400
200
0
0 10 20 30 40 50 60 70
g
ADULT
1000
800
600
400
200
0
0 10 20 30 40 50 60 70
NUMBER OF LABELLED CELLS

Fig. 8 Graphs summarising the positions of layer 4 cells during development, after an injection of BrdU on E14, showing the positions of (a) densely labelled cells and (b) lightly labelled cells. The position of layer 4 cells was calculated by locating the modal bin (i.e. the bin with the highest number of cells) plus any bin which contained 50% of the modal bin. This is represented by the shaded area. The slightly thicker area with the filled circle indicates the modal bin. In addition, the range in position of the labelled cells outwith the shaded area is indicated by the broken line. The graph therefore, shows the position of at least 95% of the labelled cells. The pial surface is donated by the broken line above the x-axis and the ventricular surface the broken line below the x-axis. The graphs are plotted on a log-linear scale.

a


E14 BrdU INJECTION: DENSE LABEL
1000
500
0
-400
DEPTH (um)
CTX/WM
BORDER
1
2
3
4
5
6
E16
E18
P0
P7
P21
AGE (days)

b

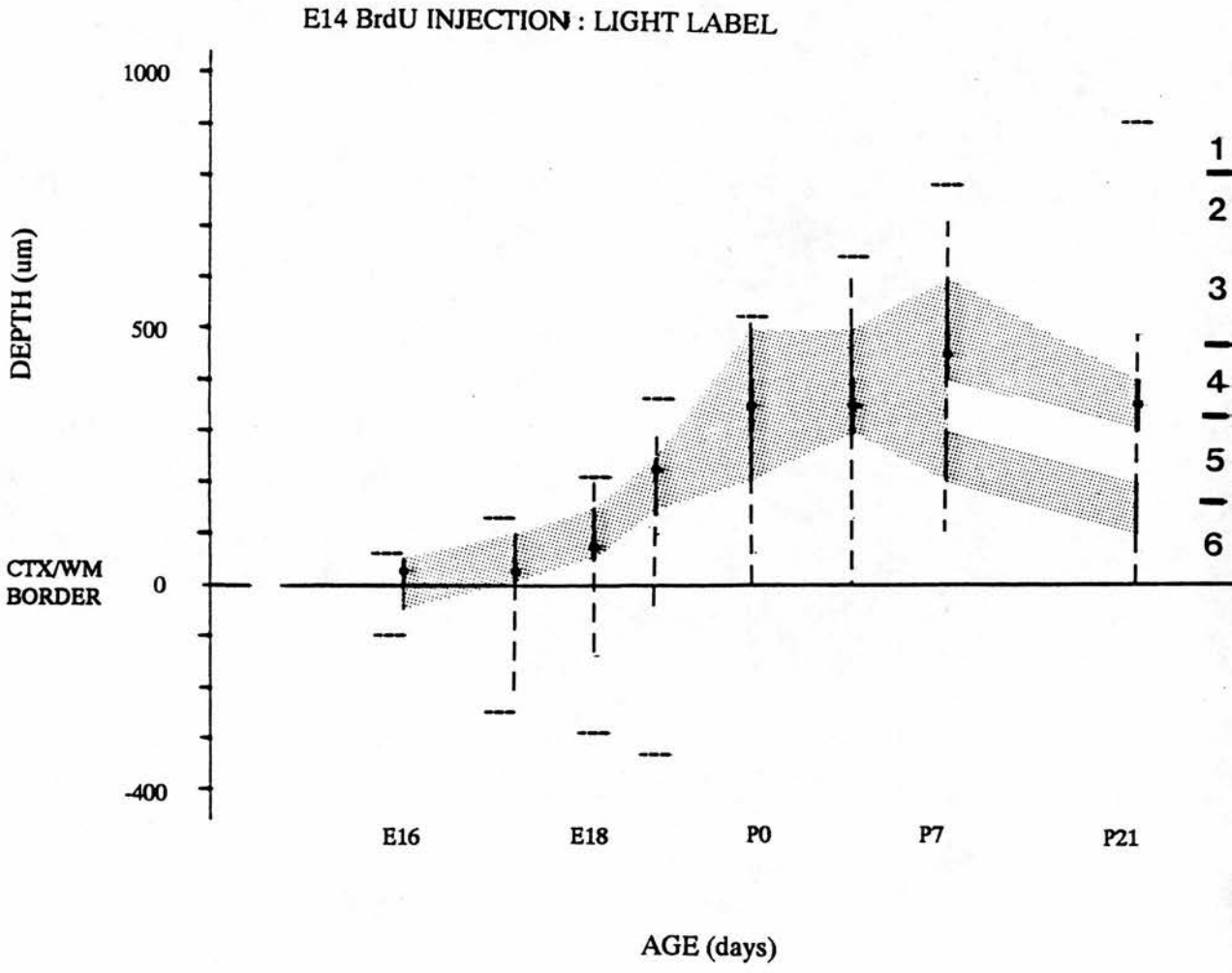
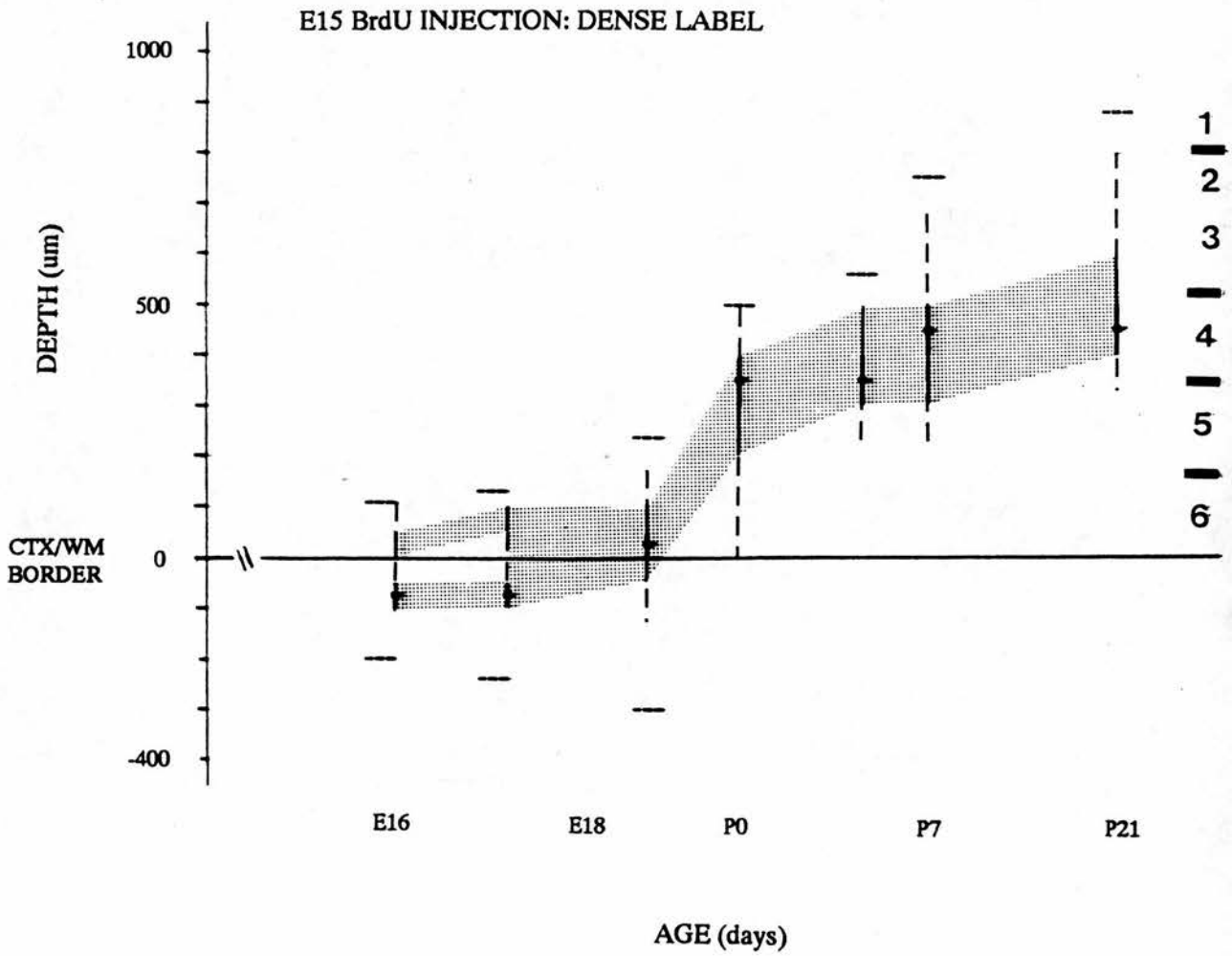
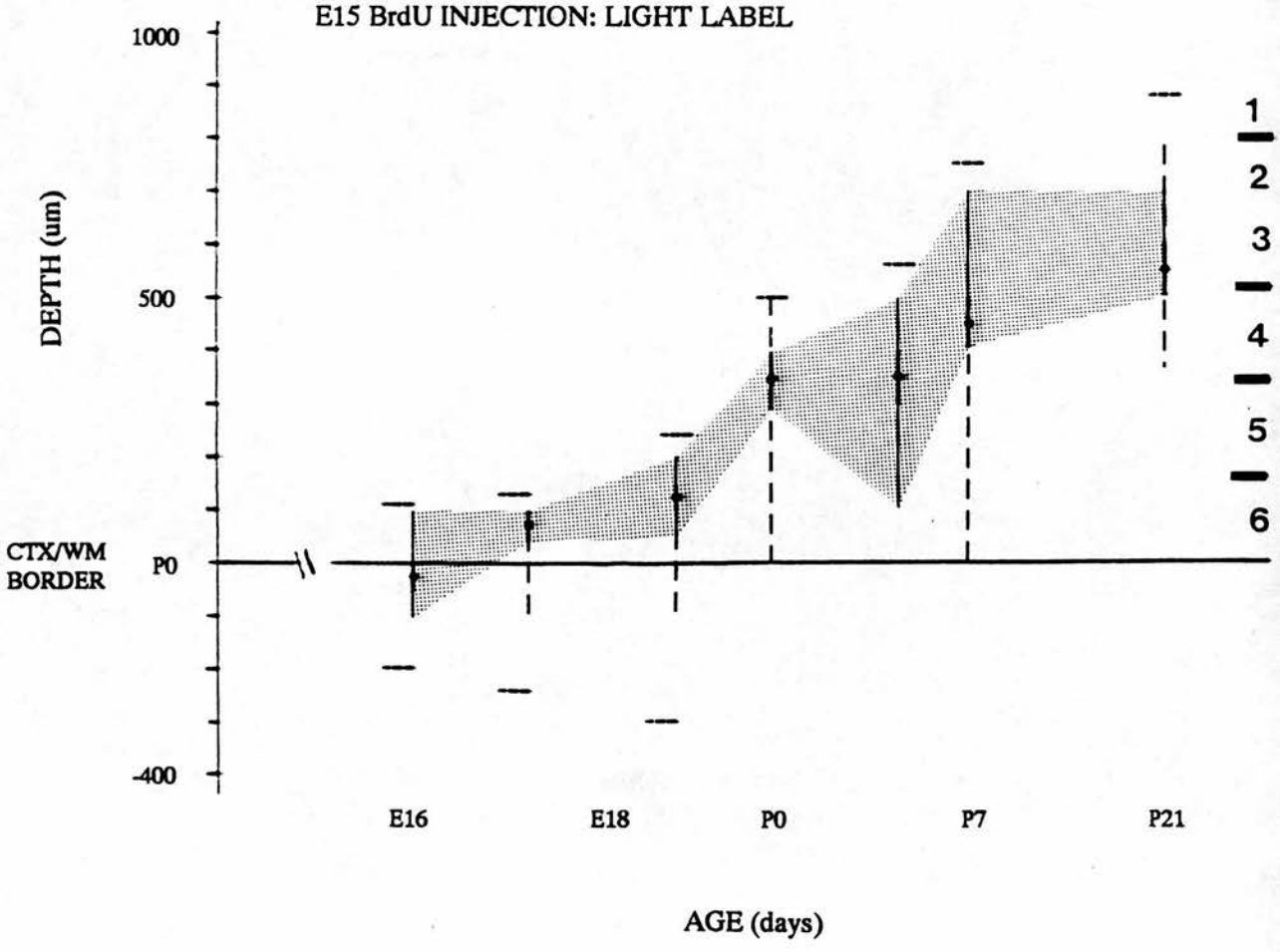

E14 BrdU INJECTION : LIGHT LABEL
1000
500
0
-400
DEPTH (um)
CTX/WM
BORDER
E16
E18
P0
P7
P21
AGE (days)
1
2
3
4
5
6

Fig. 9 Graphs detailing the position of layer 4 cells after injection of BrdU on E15: (a) the postions of densely labelled cells and (b) the positions of lightly labelled cells. The conventions are as in Fig 8.

a


E15 BrdU INJECTION: DENSE LABEL
1000
500
0
-400
DEPTH (um)
CTX/WM
BORDER
1
2
3
4
5
6
E16
E18
P0
P7
P21
AGE (days)

b


E15 BrdU INJECTION: LIGHT LABEL
1000
500
P0
-400
DEPTH (um)
CTX/WM
BORDER
E16
E18
P0
P7
P21
AGE (days)
1
2
3
4
5
6

To have some overall method of presenting data on cell migration, the following scheme was adapted. Figs. 6 and 7 were used to determine the bin where the highest number of cells were located (the mode). This was the position of the highest density (in some cases most) of the layer 4 cells at that time. In addition, any bin which contained at least 50% of the modal number of labelled cells was also noted. This step was carried out because often two (or in some cases 3) bins had a similar number of cells. To have discarded these bins would not have presented a true reflection of the position of layer 4 cells. For ease of analysis and presentation, the positions of lightly and densely labelled cells were plotted separately.

The progress of cells born on E14 is described in Fig. 8. Fig. 8a details the position of the densely labelled cells. It is apparent that layer 4 cells are already present in the cortical plate on E16. Their position remains fairly constant at the lower edge of the cortical plate until E19. The reason for this delay in migration is unclear. Fig. 8b shows that the lightly labelled cells have already began to migrate beyond the densely labelled cells. It may be that the densely labelled cells wait until the lightly labelled cells pass them (Fig. 8b), and then the two populations migrate into their final positions together. The two populations of cells overlap slightly and appear to migrate together until around P3. At E19 the cells begin to migrate further into the cortical plate with most cells located between 50-150um from the cortex/white matter border. On the day of birth, all labelled cells are present in the cortical plate and have migrated to take up positions 100-300um from the border of the cortex. On P3, labelled cells are present in a large band 100-400um above the cortex/white matter border. By P7 the width of the band has increased to occupy a zone from 100-500um above the cortex/white matter border. The band of cortex covered by labelled cells on P3 and P7 resembles that of the adult. The band in the adult

however, is slightly narrower. This may be a result of differentiation of cortical cells and the fine tuning of migration.

The migration of lightly labelled cells after an injection of BrdU on E14 (Fig. 8b) follows a similar pattern to that of the densely labelled cells. It should be noted that the lightly labelled cells take up slightly more superficial positions than the densely labelled cells. This is true of lightly labelled cells from around E17. However, at E17 until around adult the two populations of cells overlap slightly. In adult the lightly labelled cells appear to split into two bands which are separated by a 100um band of unlabelled cells. These bands (both that of the unlabelled and labelled cells) occupy a similar position to the band of densely labelled cells (see Fig. 8a). It is unclear why this occurs as lightly labelled cells almost always occupy more superficial positions in the adult. It may be that the BrdU reaction with this animal was less sensitive than in others (see section 2.4, Discussion). This being the case the lower band of lightly labelled cells may actually belong to the densely labelled catagory of cells but has been grouped with the lightly labelled cells.

The migration of cells labelled with BrdU on E15 is presented in Fig. 9. Fig 9a describes the migration of densely labelled cells. A small number of labelled cells are located in the cortical plate on E16; most of the labelled cells, however, are still in the intermediate zone. On E19 most of the labelled cells have migrated into the cortical plate, however, some cells are still located in the intermediate zone. These cells are confined to the upper part of the intermediate zone. By P0 (the day of birth) all the cells have migrated into the cortical plate and are situated between 200-400um above the cortex/white matter border. By P4 they are in a position which resembles that of the adult. The E15 densely labelled cells occupy a slightly more superficial positions than those of E14 densely labelled cells.

The position of lightly labelled E15 cells is shown in Fig. 9b. These cells followed a similar pattern of migration to the above. However, the final positions of lightly labelled cells was more superficial than those which were densely labelled and was the most superficial of all labelled cells.

2.3.3 Summary

The results presented in this chapter indicate that injections of BrdU will successfully label cells born on the day of injection. The migration of the labelled cells can subsequently be monitored.

Cells labelled on both E14 and E15 are present in the cortical plate on E16, although a large number of these cells are located in the intermediate zone. Their migration into the cortical plate is complete by birth. The layer 4 cells begin to take up their final positions around P3, however, it is apparent that significant changes occur after this time.

Lightly labelled cells migrate beyond the densely labelled cells to more superficial postions in almost every example presented here.

2.4 *DISCUSSION*

In this chapter I have investigated the birth of cortical layer 4. As the target of ingrowing geniculate axons, the fate of the cells destined to form this layer was an important event which I needed to establish the timing of. This information was important so that I could culture a slice of occipital cortex secure in the knowledge that the target cells were present in the slice.

The results presented in this chapter indicate that layer 4 cells first enter the cortical plate on E16. They continue their migration into the cortical plate

until around the day of birth at which time all cells destined for layer 4 are present. However, the layer 4 cells are not in their final positions at birth; rather, they are distributed throughout the bottom half of the cortical plate. It is not until around P3 that the cells are in their final positions (although some fine adjustments still occur until after P7).

The fact that layer 4 cells are in position around P3, although they are not fully in position until later, is consistent with the observations in the rat (Berry and Rogers, 1965; Lund and Mustari, 1977). The first geniculate axons destined to form synapses with layer 4 cells are observed in the cortical plate around E17 (see chapter 3, Development of the lateral geniculate nucleus and the formatiom of the geniculocortical pathway). This event is consistent with the early migration of layer 4 cells into the cortical plate.

BrdU was used in this study for several reasons. Firstly, this was a convenient method of labelling cells as the molecule can cross the placental barrier, thus labelling fetal cells. Secondly, the half-life of this molecule is quite short (around 12 hours). As a result, I was able to inject the pregnant mouse and be fairly certain that only cells born on that day would be labelled. That is , it is unlikely that there would be any residual BrdU available to label cells dividing on subsequent days. The immunohistochemical reaction which allows the identification of labelled cells is a fairly simple procedure, with data available after only a few hours (tritiated thymidine experiments can take up to 8 weeks).

Labelled cells fell into two catagories, those that were densely labelled and those that were lightly labelled. I made a distinction between these two catagories of cells based on the observations of Smart and Smart (1982). It was likely that densely labelled cells may be the product of the first round of cell division, while lightly labelled cells are the product of a second or even a third

round of cell division. It may also be that lightly labelled cells are cells entering s-phase at the end of the pulse of BrdU or cells ending s-phase as the BrdU is given.

One major disadvantage of this technique is illustrated by the position of lightly labelled cells analysed on P4 after an E15 injection. These cells appeared to occupy almost the entire thickness of the cortex (Fig. 9b). This may have been an artefact of analysis. The labelling of cells was often borderline, making it difficult to differentiate between light and dense label. In these cases I tended to identify the cells as lightly labelled. There seems to be no way round this difficulty. Increasing the magnification under which sections were analysed helped slightly, although there were still some cells that could not be categorically differentiated.

CHAPTER 3

DEVELOPMENT OF THE LGN AND THE FORMATION OF THE GENICULOCORTICAL PATHWAY

3.1 *INTRODUCTION*

The lateral geniculate nucleus (LGN) is one of many relay nuclei of the thalamus (see section 1.3.2, The LGN). The retina transfers information via the LGN to the visual cortex.

In this chapter I have investigated two facets of LGN development. Firstly, the date on which cells of the LGN were born was investigated. Here I used the BrdU labelling technique described and discussed in chapter 2. Secondly, I investigated the formation of the pathway between the LGN and the visual cortex (the geniculocortical pathway). For this series of experiments I used the carbocyanine dye, DiI, to trace the formation of the pathway in fixed tissue.

Both of these pieces of information were of great importance to the experiments presented in chapters 4 and 5. It was important to determine that when the LGN explants were collected for culture, all the constituent cells were present. Also it was useful to know how developed the pathway was when the explants were collected.

3.2 *MATERIALS AND METHODS*

3.2.1 Animals and BrdU injections

BALB/c mice from an isolated laboratory colony were mated overnight. On the following day (deemed to be embryonic day 1 E1) mice with a vaginal plug were removed from the colony. The pregnant mice were injected with a single dose (0.2mls) of BrdU dissolved in sterile saline on E12-E16. This injection was made i.p., with a solution of 2mg/ml BrdU.

3.3.2 Preparation of tissue after BrdU injection

Tissue was collected from animals on P21 (at this time the cortex has a similar appearance to that of the adult). Mice were killed with an overdose of sodium pentobarbitone (0.3mls at 12mg/ml, i.p.). These animals were then perfused trancardially with saline and then 4% paraformaldehyde in phosphate buffer. The brains were then post-fixed with a similar concentration of fixative for 1 hour. The tissue was then placed in phosphate buffered sucrose (with 20% sucrose) overnight. 30um parasagittal sections were cut using a freezing microtome. The sections were then mounted onto glass slides pre-coated with 0.01% poly-L-lysine.

3.3.3 Immunohistochemistry

Sections were reacted following the procedure described in chapter 2, section 2.2.3, Immunohistochemistry.

3.3.4 Analysis

The immunochemical technique described in section 2.2.3 was used to identify cells born on E12-16. BrdU labelled nuclei appear dark brown and are easily identified from the surrounding unlabelled cells. Only labelled cells located in the LGN were analysed. Camera lucida drawings of adjacent sections of LGN were made and the numbers of labelled cells were counted.

3.2.5 Animals and DiI injections

BALB/c mice were mated as described in section 3.2.1. The pregnant mice were sacrificed 17 days after the appearance of a vaginal plug (E17). The mother was killed by an overdose of sodium pentobarbitone (0.3mls of a 12mg/ml solution i.p.). The fetuses were removed and the heads were placed in

4% paraformaldehyde in phosphate buffer for 24 hours. The brains were dissected out and placed in the same fixative for a minimum of 1 hour.

Using a dissecting microscope, small cuts were made over the dorsolateral thalamus and a small crystal of DiI (carbocyanine dye, dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate) was inserted into the cut. The brains were then returned to the fixative and stored in the dark at room temperature for at least 1 month to allow the DiI to diffuse (Godement et al., 1987).

3.2.6 Preparation of tissue DiI injection

The brains were removed from fixative and placed in egg yolk. The egg was vapour fixed with 25% formaldehyde in phosphate buffer at 37^{o}C for 24 hours, then placed in 10% formalin in phosphate buffer at 4^{o}C for a further 24 hours. The fixed egg yolk, containing the DiI injected brain, was then blocked and 30um sections were cut using the freezing microtome. The sections were then mounted onto glass slides and viewed using a fluorescence microscope.

3.3 *RESULTS*

3.3.1 Birth dating the LGN

The BrdU label was confined to the nucleus of the cell. The labelling of cells varied in intensity, see chapter 2. However in this study the intensity of label was not judged to be important, as I was not looking at the migration of cells, simply at the presence or absence of label.

The number of labelled cells found in the LGN of each section was

counted. The area of the nucleus was calculated. This allowed me to obtain the density of labelled cells (Fig. 1).

Fig. 1 details the denisty of labelled cells found after injection of BrdU on E12-16. It appears that most of the geniculate cells are generated on E12-13, with only a small number of cells being born on E14. No labelled cells are observed on either E15 or E16. From these results it is clear that the birth of the LGN begins before E12 and is complete by E15.

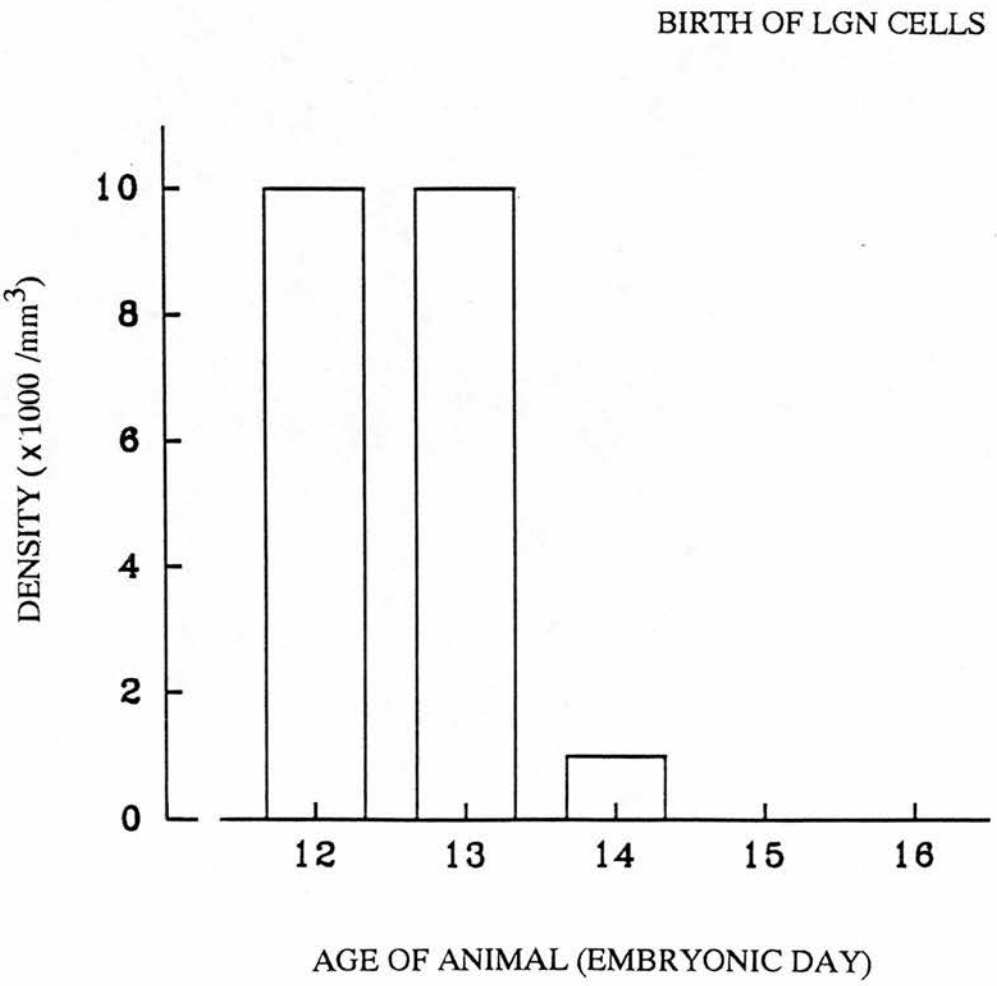


Fig. 1 Histogram showing the density of labelled cells located in the LGN on E12-16.

3.3.2 Growth of geniculate axons to the cortical plate

The growth of geniculate axons towards the cortex was investigated. DiI diffuses slowly in the plasma membrane of fixed cells. Here a crystal of DiI was inserted into the LGN on E17 and the trajectory of the axons of the geniculate cells could be observed. Fig. 2 shows a slice of E17 cortex (embedded in egg yolk). DiI labelling was observed in the cortical plate. This implies that the geniculocortical fibres have already reached the cortical plate by E17. The fibre tract is not seen in Fig. 2. It was labelled, and observed in sections more rostral than this. The injected LGN was also seen in the more rostral sections.

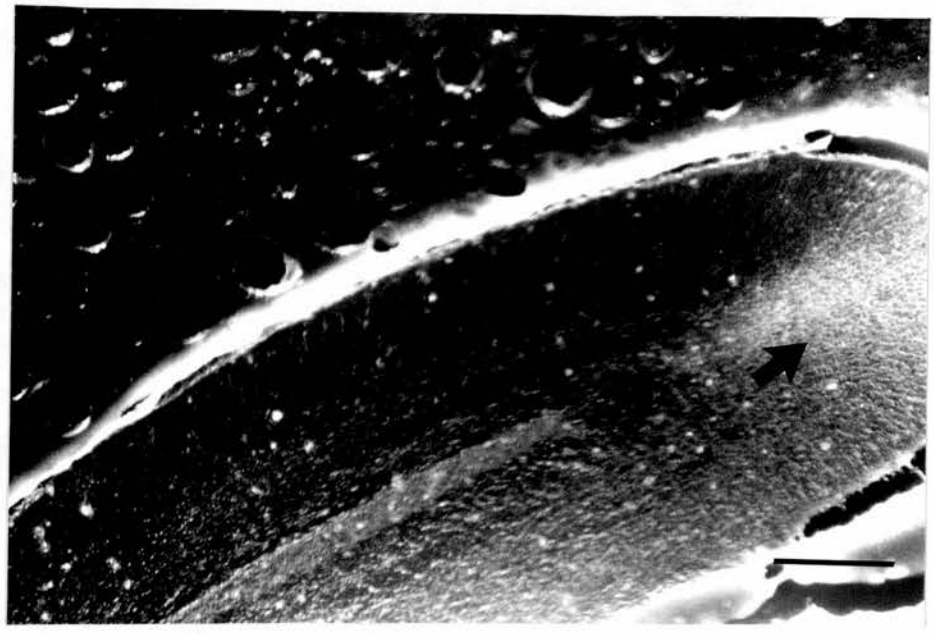


Fig. 2 Photomicrograph of a slice of visual cortex on E17. DiI was placed in the LGN and labelling is observed as the bright region at the posterior end of the telencephalon (indicated by an arrow). Scale bar, 200um.

3.4 DISCUSSION

The birth of the murine LGN is complete by E14. This is similar to the birth of the LGN in the rat; Lund and Mustari (1977) found that the LGN is born between E12-14 in rat.

The main reason for undertaking this study was to find out if all the LGN cells were born when LGN explants were cultured (see chapters 4 and 5). Explants were collected for culture on E16. As no labelled LGN cells were found on either E15 or E16, it is certain that all geniculate cells were born by the time explants were collected for culture.

The growth of fibres from the LGN to the visual cortex was also investigated. It was apparent that the geniculate fibres had reached the cortical plate by E17. This observation agrees with those of Catalano et al. (1989) and De Carlos and O'Leary (1992) in the rat. De Carlos and O'Leary (1992) state that the thalamocortical projection has reached the cortex as early as E16 in rat.

I attempted to expand this study to include periods both before and after E17. However, I did not succeed with these experiments. Although DiI is very successful in labelling and identifying cells and fibre tracts, it is extremely sensitive to organic solvents. The pathway I was attempting to label is established at a very early stage. This means that the brain is still quite small and very delicate. For this reason it was important to support the brain by embedding it in a medium that could be cut to give approximately 30um-thick sections. Unfortunately, DiI is lost if heated and it is soluble in alcohol (making wax embedding impossible). The egg yolk used in Fig. 2, although successful to a certain degree, was inconsistent. I plan to repeat these experiments exploring other embedding mediums suggested to me by D. O'Leary and colleagues.

CHAPTER 4

IN VITRO STUDY OF GENICULOCORTICAL DEVELOPMENT

4.1 INTRODUCTION

The visual cortex receives its major afferent input from the retina via the LGN. The geniculocortical pathway runs in the optic radiation and projects to layer 4.

Some aspects of the early development of this pathway can be mimicked in vitro and organotypic culture techniques have been used by several workers to examine target recognition by thalamic neurones (Yamamoto et al, 1989 and 1992; Bolz et al, 1990 and 1992; Molnar and Blakemore, 1991; Toyama et al, 1991). For example, when explants of the embryonic LGN and the neonatal visual cortex are co-cultured, geniculocortical connections form, apparently with cells in layer 4 (Yamamoto et al., 1989; Molnar and Blakemore 1991). These methods have been used to investigate the specificity with which the cultured LGN can innervate different cortical areas (Molnar and Blakemore 1991).

Little is known about possible diffusible factors that may stimulate the growth of geniculate axons, direct thalamocortical projections towards cortex rather than to other regions of the brain, maintain their connections and underlie the plasticity of the newly formed pathways. In other regions of the developing nervous system, diffusible trophic molecules have important roles in promoting neuronal survival and growth (e.g. Lumsden and Davies, 1983 and 1986; Davies 1988). "Neurotrophic Theory" (Purves, 1988) suggests that early changes in neuronal connectivity result from competition for a limited supply of trophic substances released by target tissues. Guidance of axons may, in some systems, be achieved by chemotropic molecules, that influence the direction of movement or outgrowth of neurones (e.g. Tessier-Lavigne et al., 1988; Heffner et al., 1990).

Whereas Yamamoto et al (1989); Molnar and Blakemore (1991) and

Bolz et al. (1992) used the co-culture system to study the growth of geniculate axons into the visual cortex and their recognition of cortical layer 4, I have used the method to search for trophic interactions during the development of the geniculocortical pathway. In the first of these experiments, I cultured LGN explants either alone or with visual cortex; later I substituted different regions of the brain for visual cortex, to examine the specificity of the interactions. The LGN explants were fixed and outgrowth analysed before it came into contact with the target explant.

4.2 MATERIALS AND METHODS

4.2.1 Animals and surgery

BALB/c mice from an isolated laboratory colony were mated overnight. Plugged females were separated from the male the following day, deemed embryonic day 1 (E1). On E16, the mother was deeply anaesthetized with urethane (0.3mls of a 25% solution in normal saline, i.p.) and the fetuses were removed by caesarian section. Using a Wild dissecting microscope, 0.1-0.2mm^3 (about 0.5x0.5x0.5mm) LGN explants were cut from the dorsolateral thalamus of each fetus (the LGN was identified as described below). The LGN explants were taken on E16 since this is the time around which outgrowth from the LGN begins in vivo (Lund and Mustari, 1977; Molnar and Blakemore 1990; Catalano et al., 1991; De Carlos and O'Leary, 1992; our unpublished observations).

Most explants from the occipital cortex, and all of those from other neural regions or liver, were collected on postnatal days 2-4 (P2-4), with the day of birth deemed P0. Postnatal mice were used to obtain most of the target

tissues since, from P2 on, all the cells that form layer 4 are known to be present in the rodent cortex (Berry and Rogers, 1965; Lund and Mustari, 1977; our unpublished observations - see chapter 2). Mice aged P2-4 were deeply anaesthetized by inducing hypothermia (animals were surrounded with ice for 7 minutes). The brains were then quickly removed and selected regions were sliced coronally at 350um using a McIlwain tissue chopper; liver from P2 mice was also cut at 350um. Some cortical explants were obtained on E16; the telencephalon was removed from the thalamus and slices were cut from its caudal pole.

4.2.2 Identification of the LGN

In the embryonic mouse, the LGN is visible as a swelling on the surface of the dorsolateral aspect of the thalamus. Evidence confirming this is presented in Fig. 1. We placed small crystals of the carbocyanine dye, 1,1' dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) into the eye of E16 mice that had been fixed with 4% paraformaldehyde in phosphate buffer. Fetuses were left undisturbed in fixative at room temperature in the dark for up to 3 months (Godement et al., 1987). Each brain was then dissected and viewed with a fluorescence microscope either as a wholemount (Fig. 1) or after being cut parasagittally at 50um using a freezing microtome (Fig. 2). In sections, anterograde labelling from the eye was found in the dorsolateral aspect of the diencephalon, immediately below the caudal pole of the telencephalon (Fig. 2c,d). When this same region was viewed in wholemounts from which the hemispheres had been removed (Fig. 1a,b), the LGN was clearly visible and its position could be related to distinctive landmarks (Fig. 1c,d). As a final check, I used a dissecting microscope to remove the region indicated as LGN in Fig 1(d) from DiI labelled brains, and then confirmed with fluorescence microscopy that

it contained label with an appearance similar to that in Fig. 1(b).

It must be recognised that, although I was confident that my explants of dorsolateral thalamus contained mainly LGN (I refer to them as "LGN explants"), it is likely that small amounts of surrounding tissues were also included (see Discussion, section 4.4).

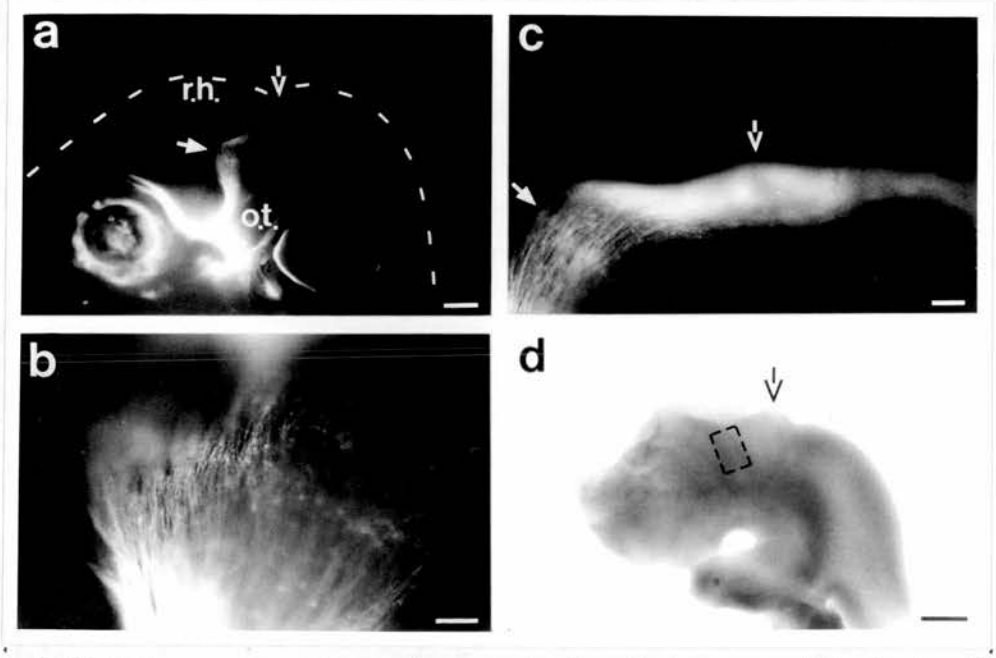
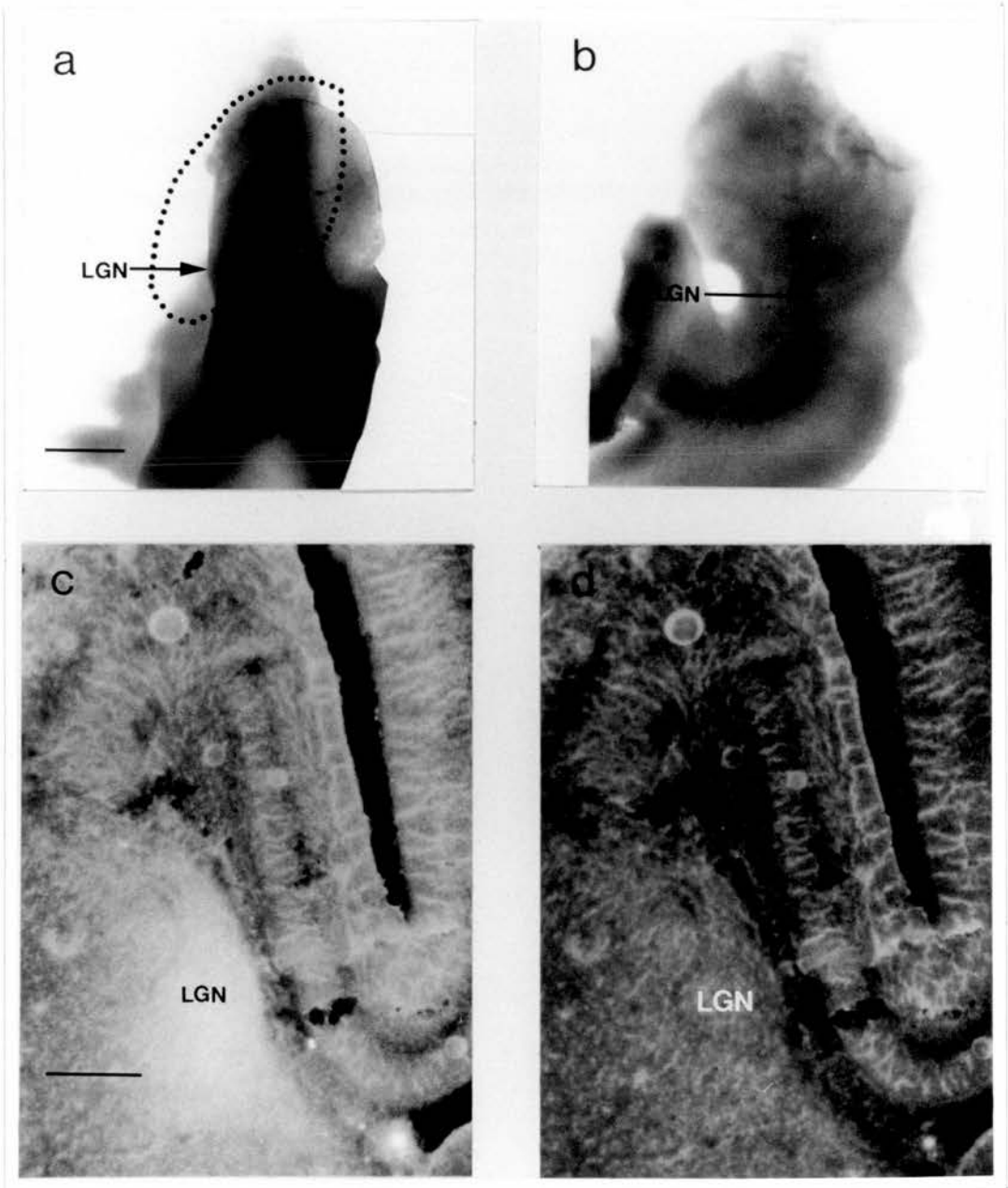


Fig. 1 (a) A fluorescence photomicrograph of a wholemount of the brain of an E16 mouse, viewed from the left side. The back of the skull and the left hemisphere have been removed; the right hemisphere (r.h.), the front of the skull and structures surrounding the eyeball remain intact. The broken white line indicates the dorsum of the brain. A crystal of DiI was placed in the eye three months before dissection. The tracer has been transported via the optic tracts (o.t.) to the LGN (filled arrow) and to the optic tectum (open arrow). (b) A high-power view of the LGN in (a), showing the spreading of DiI labelled retinogeniculate axons as they emerge from the ventral diencephalon. (c) The brain in (a) has been removed from the skull, sliced along the midline and laid on its cut surface, as if in preparation for taking an LGN explant. DiI is seen in the LGN (filled arrow) and the retinotectal tract (dipping away from the plane of focus, open arrow). (d) The left side of an unlabelled, living E16 mouse brain prepared as in (c) for taking an LGN explant. The open arrow points to a consistently present and very distinctive lump which I used as a landmark (seen below the open arrow in c). Immediately rostral to this, the LGN appears as a clear swelling on the dorsolateral thalamus; the region from which LGN explants were taken is outlined. Scale bars: a & d, 500um; b, 50um; c, 100um.

Fig.2 Photographs of the left thalamus of the embryonic mouse brain, indicating the position of the LGN: (a) the brain is cut along the midline and the thalamus is exposed by removal of the overlying cortical hemisphere (former position indicated by the dotted line). The LGN appears, from this dorsal view, as a small bulge on the side of the thalamus. (b) The thalamus in (a) is turned and viewed from the lateral aspect. The LGN is clearly visible as an elongated structure protruding from the dorsolateral surface of the thalamus. (c) A fluorescence photomicrograph of a parasagittal section of the embryonic mouse brain, viewed with rhodamine optics. After placing a crystal of DiI into the eye of the fixed fetus, intense anterograde labelling (seen here as the lightest region) is observed in the LGN, just ventral to the caudal fold of the telencephalon. The background is light because of autofluorescence (demonstrated in d). (d) The photomicrograph in (c) is viewed with a fluorescein filter; there is autofluorescence of the section, allowing the boundaries of the LGN and other brain structures to be seen. The DiI labelling of the LGN is only seen with rhodamine optics. Scale bar for a and b, 750um; for c and d, 225um.


a
LGN
b
LGN
c
LGN
d
LGN

4.2.3 Culture methods

Slices of brain and liver, and LGN explants, were collected as rapidly as possible; as they were accumulated they were placed in an oxygenated balanced salts solution in an incubator at 37^0C and 5% CO_2, where they remained for no more than 40 minutes. Once sufficient material had been obtained, the tissues were removed from the incubator and arranged on a collagen coated filter (Costar UK, Transwell-col chambers with 3um pores) suspended in a chemically defined serum-free medium (Bottenstein and Sato, 1979; Romijn et al., 1988). The filters and medium had been preincubated at 37^0C and 5% CO_2 for 2 hours. LGN explants were placed on all the filters; in some cases, LGN explants were cultured alone, whereas in others occipital cortex, frontal cortex, cerebellum, medulla or liver were co-cultured at a small distance from the LGN explants. Table 1 gives the numbers of cultures and the ranges of distance between explants (measured after culture, see below). 2mls of medium were placed in the lower chamber of each culture-well and 200-250ul of the same medium were placed in the upper chamber, ensuring the explants were just covered. The culture dish was then returned to the incubator at 37^0C and 5% CO_2, and remained in the incubator undisturbed for either 2 or 3 days in vitro (2 or 3 DIV, see Table 1). Over this period, each LGN explant spread slightly but maintained its original shape; the explants of cortex, cerebellum and medulla also maintained their shape, but with less spreading. It has been shown previously that cultured tissue explants keep their characteristic organisation (Gahwiler, 1988; Yamamoto et al., 1989 and 1992; Bolz et al., 1990 and 1992; Molnar and Blakemore, 1991). Once the culture period was complete, the medium was replaced by a similar volume of 4% paraformaldehyde in phosphate buffer for a minimum of 2 hours to fix the tissue.

4.2.4 Analysis

Explants were viewed under phase-contrast using a Nikon inverted microscope. A x4 objective provided a general view, but to detect and quantify all of the outgrowth from each explant a x10 objective was necessary. Quantification was carried out using an eyepiece fitted with a calibrated graticule.

Under phase-contrast, the Transwell collagen filters appear to be grooved, with the narrow furrows (about 35um wide) all running parallel (Figs 4-6). With the scanning electron microscope, the filter is seen to comprise a lattice of fine fibres, the majority of which form regularly spaced bundles orientated in a single direction (creating the grooves), while a smaller number cross perpendicular to the grooves. This structure is created when the microporous membranes are treated with collagen, and clearly acts as a guide for fibres growing from the LGN explants. We used this invaluable property of the filters to channel the majority of the growth so that it was easy to quantify. The explants were usually arranged as illustrated in Fig. 3, with the grooves of the filter running parallel to the axis through the LGN explant and the target. With this arrangement, there was no mechanical obstruction to prevent fibres from the LGN explant growing freely either towards or away from the target. The grooves kept the majority of the outgrowth well-ordered and individual fibres and fascicles straight, making measurements of neurite length and density relatively straightforward and accurate. In most cases, LGN explants produced outgrowth that was consistent in density and length from the top to the bottom of the explant, resulting in a fairly even neurite front. As shown in Fig.3, growth was quantified on each side of the LGN explant within three regions whose centres were opposite (i) the midpoint of the LGN explant in the plane perpendicular to the collagen grooves (point M), (ii) a point halfway between M

and the upper edge of the explant (point T), and (iii) a point halfway between M and the lower edge of the explant (point B).

The length of the culture period (2 or 3 DIV) was deliberately too short to allow physical contact to be established between the explants. Although I often observed outgrowth from the target tissue, it was much shorter than, and did not overlap, that arising from the LGN explants. The distances parallel to the collagen grooves between the edge of the explant and the neurite front opposite points M, T and B were measured (Fig. 3), and the average length of the neurite front was calculated separately for growth towards and away from the target. The length of the longest single neurite along the entire front was always noted; this was not used in the calculation of average length of neurite outgrowth (it was always in proportion to the average distance from the edge of the LGN explant to the neurite front).

A semi-quantitative method was used to estimate the density of neurites growing from the explant; as for neurite length, data on growth away from the target was kept separate from that on growth towards the target. On each side of the LGN explant, three 200um-wide windows were drawn perpendicular to the collagen grooves, with their centres opposite points M, T and B, as shown in Fig. 3. The densities of neurites at the explant edge within these windows were estimated on a scale of 0-10, and average values both towards and away from the target were obtained. This method was adopted because it was often too difficult to count the absolute numbers of neurites emerging from each explant. In making these relative assessments of neurite density repeated counts were made only then were they converted to the ranking scale. A test to determine the interobserver error showed that there was a 13% interobserver error. Where bundles of axons, or fascicles, were observed I had to rely on an estimate of the numbers of neurites based on the widths of the fascicles. The resolution of a 0-

outgrowth.

The area of each explant was estimated, as was the distance between them (expressed as the average of three measurements of separation taken at equally spaced positions along the gap; Table 1). These measurements were taken after completion of the culture period, and took into account spreading of the tissue.

Statistical analyses were carried out using a Wilcoxon rank sum test on unpaired data; this test makes no assumption about the nature of the distribution of data.

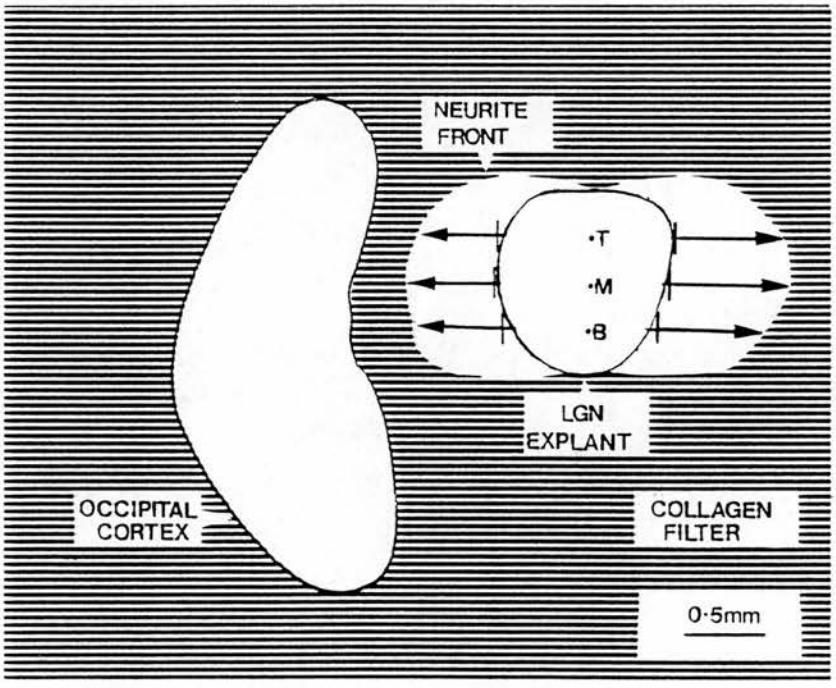


Fig.3 Schematic diagram illustrating an explant co-culture and the method of quantifying outgrowth. The parallel horizontal lines indicate the orientation of the collagen grooves on the filter (seen in Figs.3-5). Three reference points on the LGN explant were used for quantification: M (midway between the top and the bottom of the LGN explant), T (midway between the top of the LGN explant and point M) and B (midway between the bottom of the explant and point M). Opposite each point, I measured the density of neurites crossing 200um windows at the edge of the explant (short vertical lines) and the length from the edge of the explant to the neurite front (arrows).

4.2.5 Labelling with antineurofilamentantibody

The fixed explants were washed twice with phosphate buffered saline (PBS) at pH7.2 (10minutes), twice with 0.05% polyoxyethylenesorbitanmonolaurate (Tween 20) in PBS (10 minutes) and then twice with PBS. The tissue was incubated with antineurofilament monoclonal antibody (Affiniti Research Products, no. NA1223, diluted 1:50 with PBS containing 0.1% bovine serum albumin) for 24 hours at $4^{O}C$, and then for a further 2 hours at $37^{O}C$. The explants were then washed thrice with PBS (10 minutes) and incubated with a fluorescein-conjugated goat anti-mouse secondary antibody (1:5 dilution in PBS) for 24 hours at $4^{O}C$, then for a further 2 hours at $37^{O}C$. The explants were washed twice more with PBS and viewed with a Nikon inverted fluorescence microscope.

4.3 RESULTS

4.3.1 Outgrowth from LGN explants either with or without target tissues

The results were from 186 cultures (Table 1). An example of an LGN explant cultured alone is shown in Fig.4; only 14% of such cultures showed any outgrowth onto the collagen filter, and in these few cases growth was very sparse. However, as shown in Fig. 5, if a slice of occipital cortex was cultured close to the LGN, profuse outgrowth was observed. Neurites grew out of the LGN explant both towards and away from the occipital cortex (examples of individual neurites or fascicles are indicated by arrows in Fig. 5). The growing neurites had a strong tendency to follow the grooves on the collagen filter. A minority of neurites, particularly those emerging from the top and bottom of the

LGN explant, did not lie parallel to the grooves (examples are seen Fig. 5a). By adjusting the plane of focus at high power, I found that the majority of these neurites were floating above the filter. Sometimes, where they came back into contact with the filter, they deviated to follow the grooves. Growth that did not follow the grooves was always shorter and less dense than that which did, and so was always covered by our quantitative assessments of outgrowth.

Explants	Ages	DIV	No.	Range of Distances (mm)
LGN	E16	2	22	
LGN + Occ. Ctx.	E16+P2	2	8	0.53-2.07
LGN + Occ. Ctx.	E16+P2	3	12	0.39-1.99
LGN + Occ. Ctx.	E16+P3	2	19	0.37-2.35
LGN + Occ. Ctx.	E16+P3	3	12	0.57-2.28
LGN + Occ. Ctx.	E16+P4	2	12	0.88-2.43
LGN + Frn. Ctx.	E16+P2	2	27	0.19-2.40
LGN + Cerebellum	E16+P2	2	42	0.67-2.46
LGN + Medulla	E16+P2	2	8	1.67-2.42
LGN + Liver	E16+P2	2	4	2.26-2.71
LGN + Cortex	E16+E16	2	20	0.56-2.01

Table 1. The co-culture experiments are listed below. The number of cultures and the range of distances between the explants are given (note that measurements of separation were made after the period of culture, and hence after any spreading of the explants). Abbreviations, DIV: days in vitro; Occ. Ctx.: Occipital Cortex; Frn. Ctx.: Frontal Cortex.

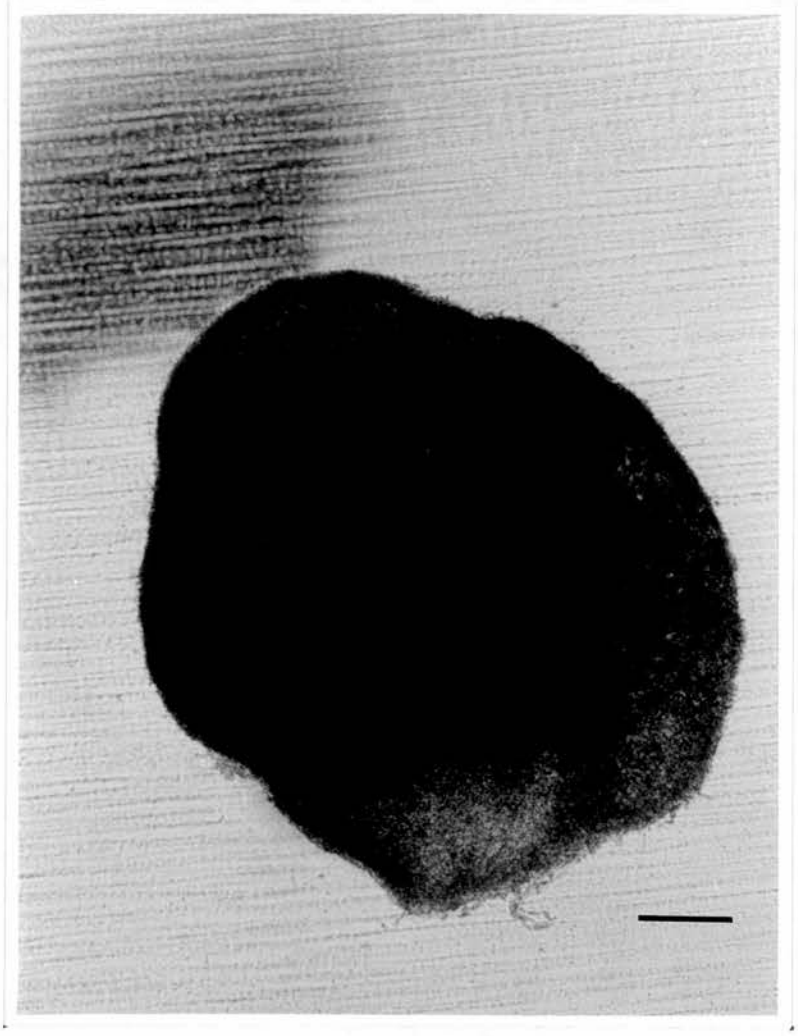
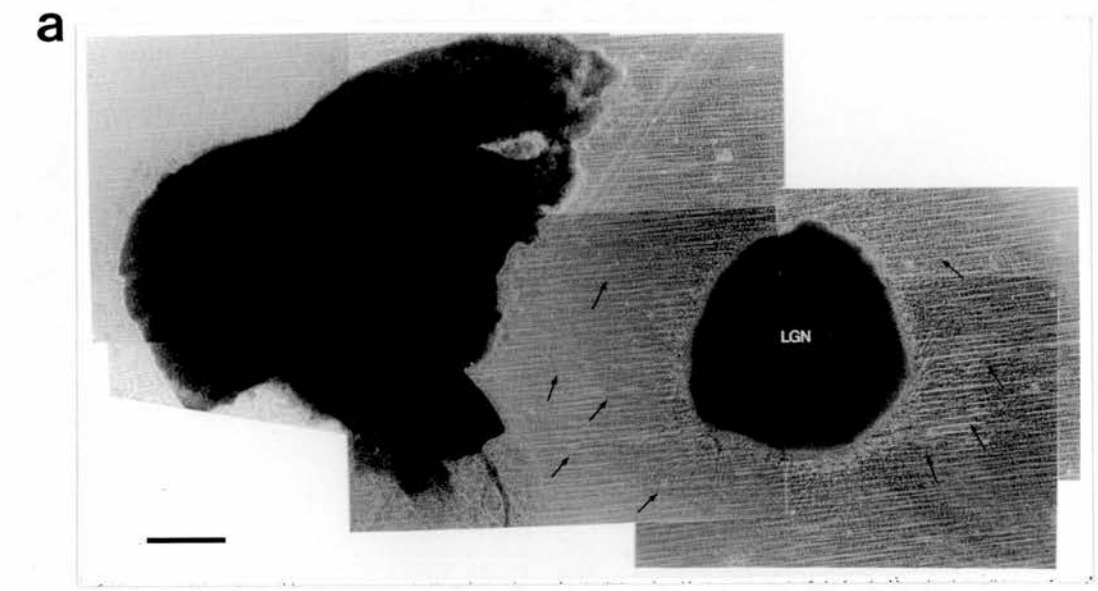


Fig.4 Phase-contrast photomicrograph of an LGN explant taken on E16 and cultured alone for 2 days; there was no outgrowth from this explant, although the majority of neurones within it appeared healthy with light and electron microscopy (see chapter 4, section 5.3.2 Cellular content of LGN explants). Scale bar, 250um.

Fig.5 Phase-contrast photomicrograph showing a co-culture of an E16 LGN explant with a slice of P2 occipital cortex, after 3 DIV. (a) A photomontage of both explants at low power. Outgrowth can be seen crossing the filter (arrows point to examples of individual neurites). Much of the longest growth follows the grooves both away from and towards the cortical explant, almost but not quite reaching it. (b) Higher power photomicrographs showing outgrowth towards and away from the cortical explant (arrows indicate examples of neurites or fascicles). Scale bar for a, 500um and b, 250um.


a
LGN

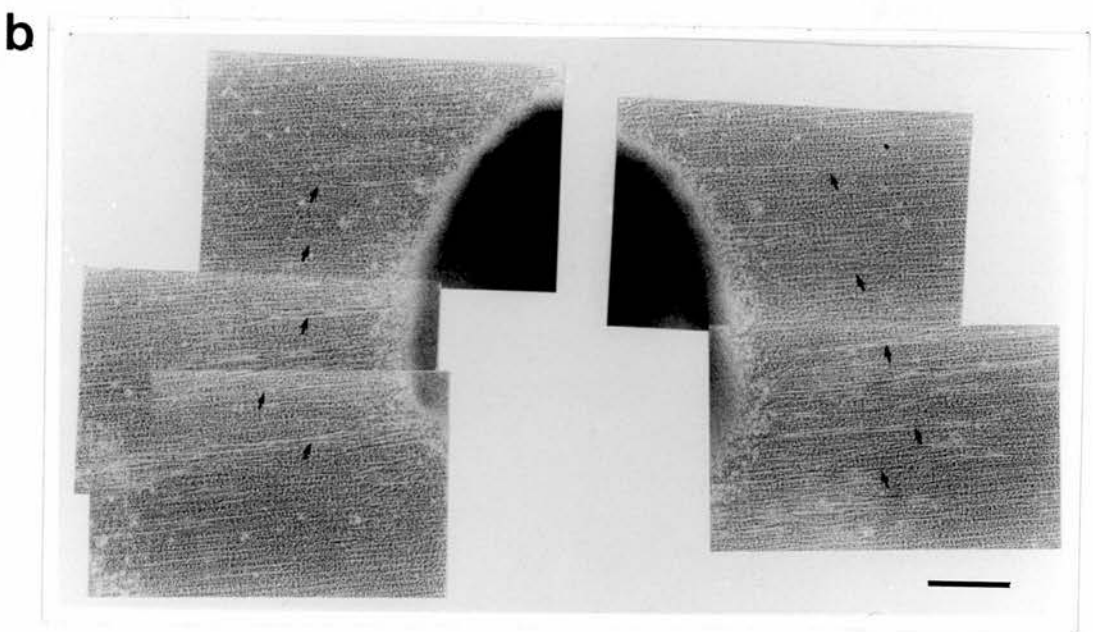
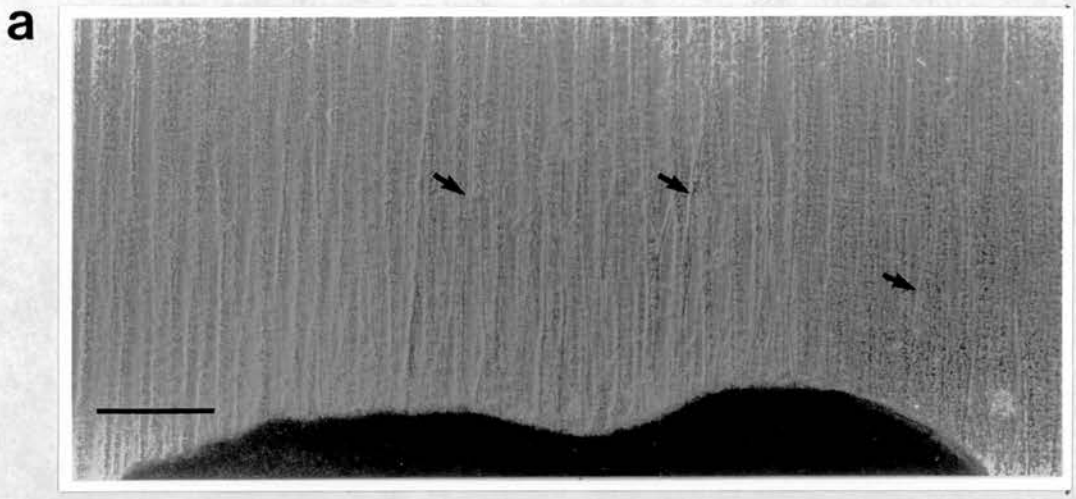
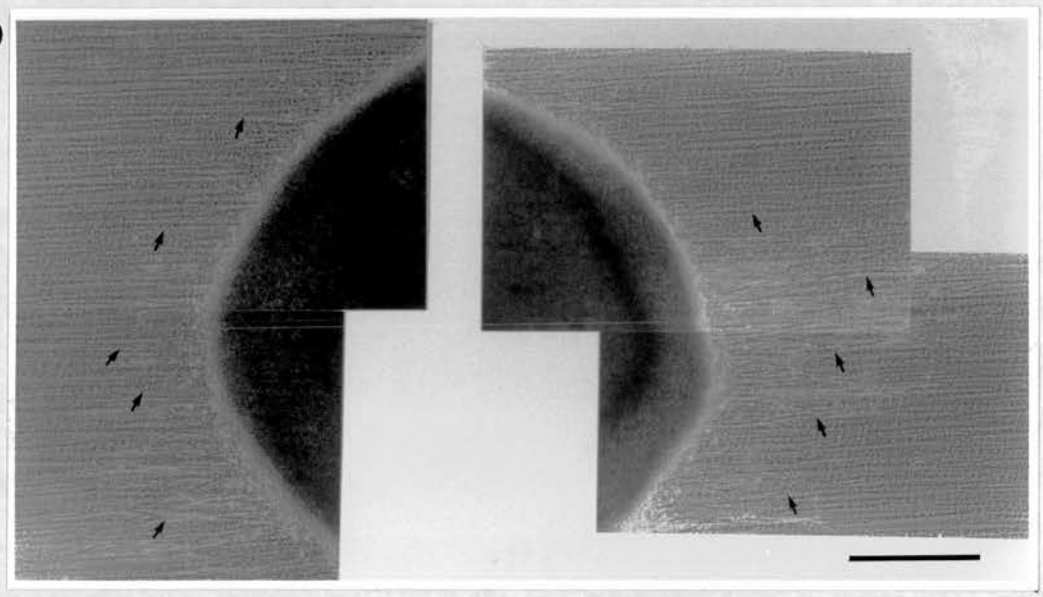
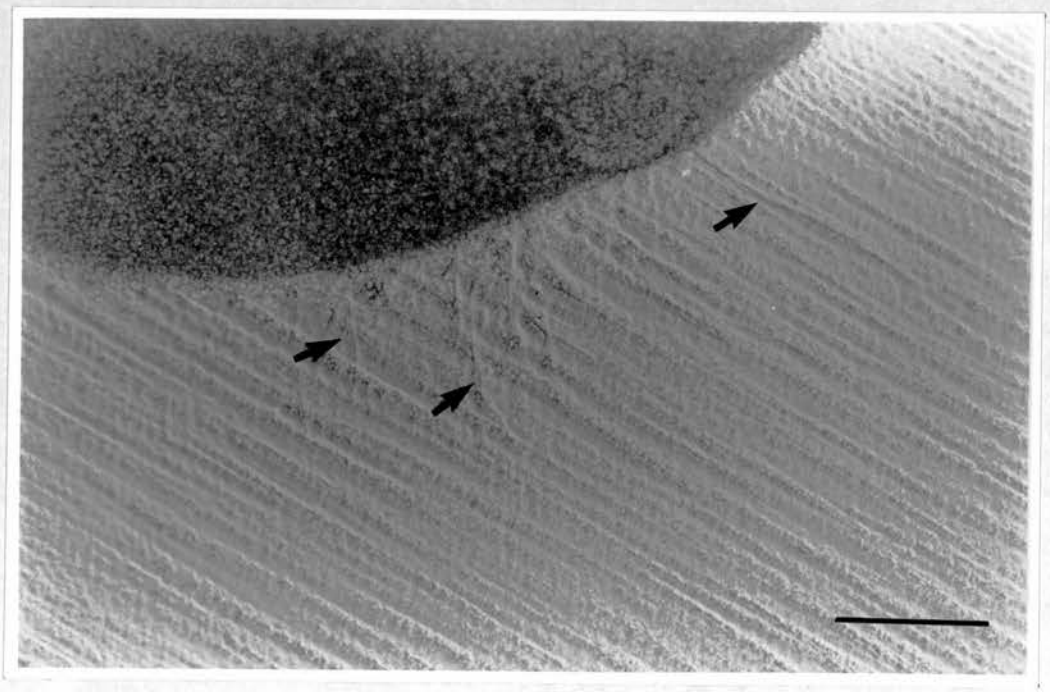

b

Fig.6 Phase-contrast photomicrographs of outgrowth from LGN explants cultured with (a) P2 frontal cortex, (b) P2 cerebellum or (c) P2 medulla. Arrows indicate examples of individual neurites or fascicles. Scale bar for a and c, 100um; b, 250um.


a


b


c

Occipital cortex from P2 animals was the most reliable stimulant, and generated profuse outgrowth from LGN explants in 100% of cases (cultured for 2 or 3 days). With older explants of occipital cortex and with other tissues there were more failures. The LGN explants produced neurites in 77% of cases when they were cultured with P3 occipital cortex for 2 or 3 DIV, and in 42% of cultures with P4 occipital cortex (for 2DIV). When the P2 occipital cortex was replaced with P2 frontal cortex, cerebellum or medulla, the percentage of LGN explants showing outgrowth after 2 DIV fell from 100% to 85% with frontal cortex, 62% with cerebellum and 50% with medulla. Examples of outgrowth with frontal cortex, cerebellum and medulla are shown in Fig. 6; growth with medulla was very sparse (Fig. 6c). When LGN explants were co-cultured with P2 liver, no outgrowth was observed from any explant.

Quantified data on outgrowth from LGN explants cultured alone or with various tissues from P2 mice are shown in Fig. 7. Neither the mean lengths nor the mean densities of outgrowth from the LGN explants differed significantly between the side towards and that away from the co-cultured tissue. The average lengths of outgrowth (indicated by the bars in Fig. 7a) were similar when the co-cultured tissue was either occipital cortex, frontal cortex or cerebellum. They were sightly lower with medulla and lowest when LGN explants were cultured alone. These values for average length of outgrowth included failures, and I decided for this, and subsequently presented data, to carry out Wilcoxon's rank sum tests on those cases where outgrowth <u>did</u> occur (i.e. ignoring failures). This analysis showed that the lengths of outgrowth from LGN explants cultured with occipital cortex, frontal cortex, cerebellum, medulla or LGN explants cultured alone were not significantly different. In other words, considering <u>only</u> those cultures where outgrowth did occur and discounting failures, I found that neurites grew to roughly equal lengths.

Fig.7 Histograms showing (a) the mean lengths of outgrowth, (b) the mean densities of outgrowth and (c) the means of the total outgrowth (mean length x mean density) from LGN explants co-cultured with P2 occipital cortex, P2 frontal cortex, P2 cerebellum, P2 medulla or P2 liver for 2 DIV. In each graph, the heights of the bars indicate average values in each experimental group; open bars are for growth towards the target, cross-hatched bars are for growth away. Individual data points for each explant are superimposed on the histograms (note that where there is more than one identical data point, they are stacked partially overlapping). In all cultures with P2 occipital cortex, there was outgrowth from the LGN explant, although in one case there was none towards the LGN explant and in another there was none away (hence the zero points).

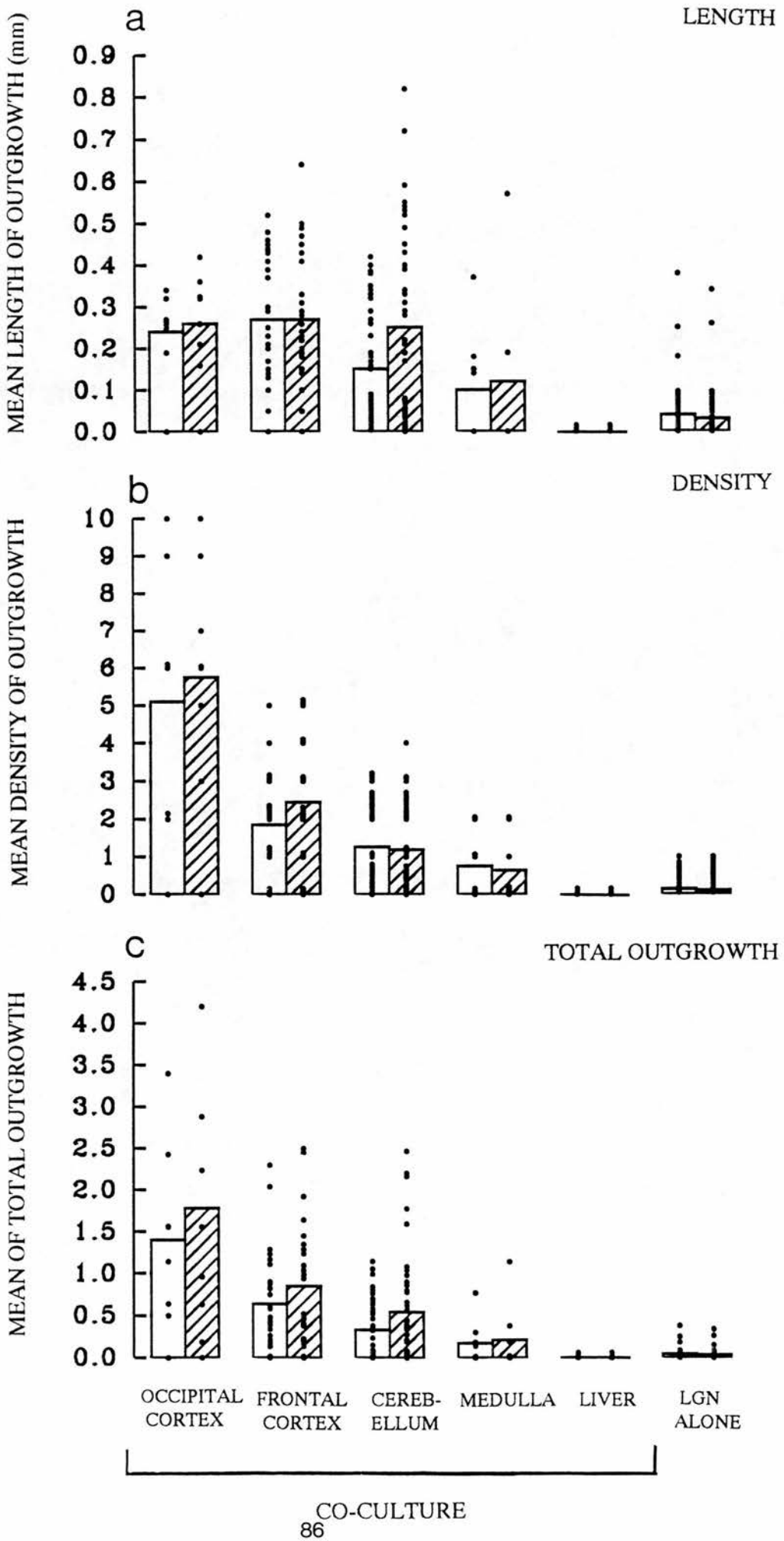
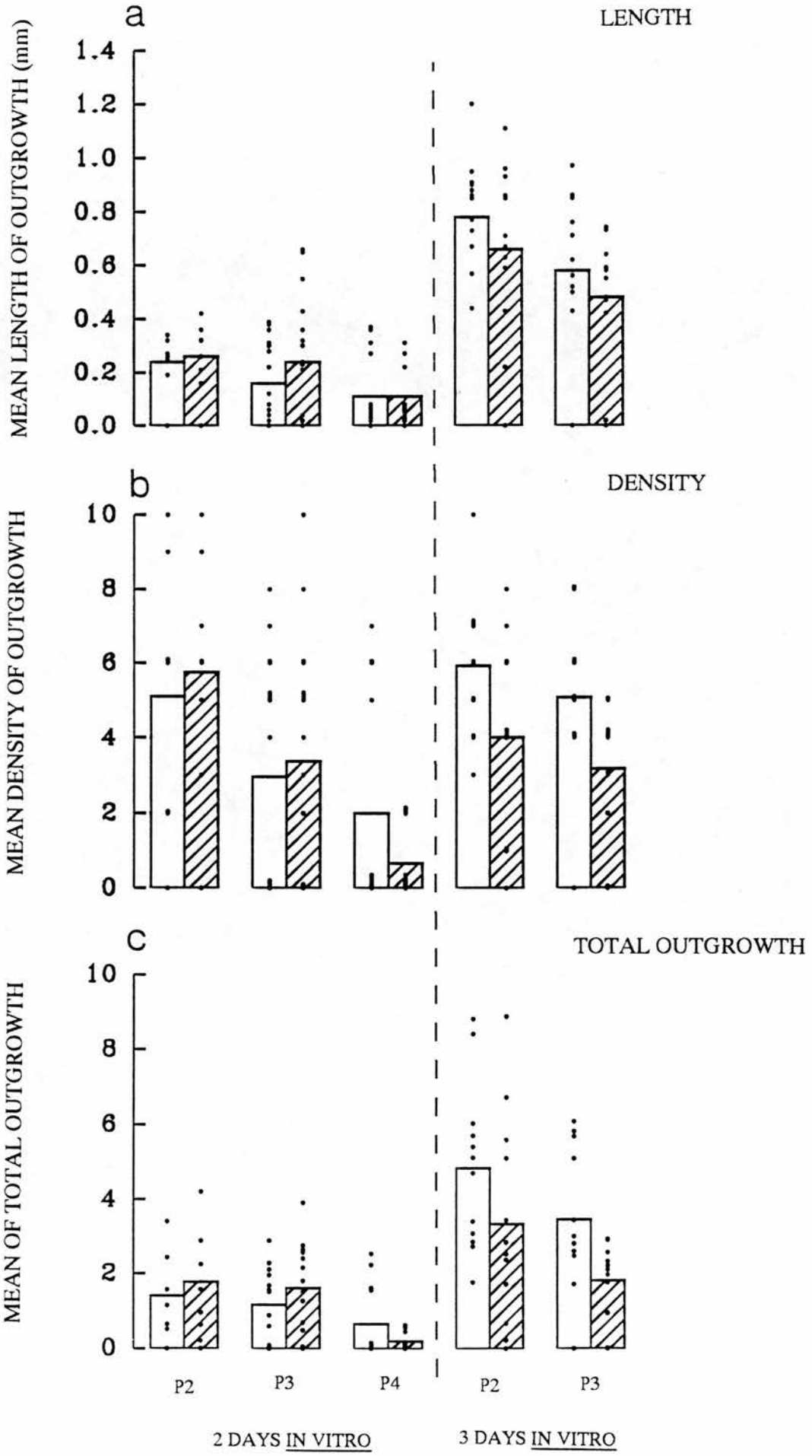

a
LENGTH
MEAN LENGTH OF OUTGROWTH (mm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
DENSITY
MEAN DENSITY OF OUTGROWTH
10
9
8
7
6
5
4
3
2
1
0
c
TOTAL OUTGROWTH
MEAN OF TOTAL OUTGROWTH
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
OCCIPITAL CORTEX
FRONTAL CORTEX
CEREB-ELLUM
MEDULLA
LIVER
LGN ALONE
CO-CULTURE

Fig.8 Histograms showing the effect on outgrowth from LGN explants of varying the age of occipital cortex and extending the culture period. (a) The mean lengths of outgrowth, (b) the mean densities of outgrowth and (c) the means of total outgrowth (length x density) are plotted. Conventions are as in Fig.6


a
LENGTH
MEAN LENGTH OF OUTGROWTH (mm)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
b
DENSITY
MEAN DENSITY OF OUTGROWTH
10
8
6
4
2
0
c
TOTAL OUTGROWTH
MEAN OF TOTAL OUTGROWTH
10
8
6
4
2
0
P2
P3
P4
P2
P3
2 DAYS IN VITRO
3 DAYS IN VITRO
OCCIPITAL CORTEX

The effects of culturing LGN explants alone or with different tissues was very pronounced when the density of outgrowth was considered (Fig. 7b). The average density of outgrowth from LGN explants (including failures; bars in Fig. 7b) was greatest with P2 occipital cortex, was progressively lower with frontal cortex, cerebellum or medulla and was lowest when LGN explants were cultured alone. Testing only those cases where outgrowth occurred (as done above for length) showed a significant reduction with frontal cortex ($p<0.01$), cerebellum ($p<0.01$) and medulla ($p<0.05$); the outgrowth from LGN explants cultured with the occipital cortex, frontal cortex or cerebellum was significantly denser than that from LGN explants cultured alone ($p<0.05$ for all tissues), but that with medulla was not.

For each explant, total outgrowth was calculated by multiplying the average neurite length by the average density on each side of the explant, to give an overall measure of the amount of outgrowth produced. The data for total outgrowth from LGN explants cultured alone, with P2 occipital cortex or other P2 tissues are compared in Fig.7(c). Total outgrowth was greatest with occipital cortex, progressively lower with frontal cortex, cerebellum and medulla, and lowest in LGN explants cultured alone. Most of the differences were significant when tested with a Wilcoxon's rank sum test ($p<0.05$; again, failures were ignored), the only exception being the difference between total growth with medulla and that from LGN explants cultured alone.

Thus, my findings were that outgrowth from E16 LGN explants was guaranteed by culturing with P2 occipital cortex, was less likely if P2 frontal cortex, cerebellum or medulla was substituted for the occipital cortex, was least likely if LGN explants were cultured alone, and was never seen if P2 liver was the co-cultured tissue. The amount of outgrowth (from the explants that did grow) was significantly reduced by the substitution of P2 frontal cortex,

cerebellum or medulla for occipital cortex, and was least when LGN explants were cultured alone. These reductions were mainly because the density of outgrowth was diminished. When outgrowth did occur, it reached similar lengths, even in the few cases where LGN explants were cultured alone. Neither the reliability of outgrowth nor its amount differed towards and away from the target.

Figure 8 shows data derived from 2DIV or 3DIV co-cultures of LGN explants with occipital cortex of different ages. There were no significant differences between the outgrowth towards and that away from the co-cultured explant in all but one set of data: the density of outgrowth away from the P4 occipital cortex after 2DIV was significantly lower than the density of growth towards the cortex ($p<0.05$; Fig. 8b).

Fig.8a shows the lengths of neurite outgrowth, Fig. 8b the densities of outgrowth, and Fig. 8c the total amounts of outgrowth. When the data were tested (ignoring the failures, which were more frequent when older occipital cortex was used, see above), the only significant differences were between the lengths and the total amounts of outgrowth from cultured explants left for 3DIV and those left for 2DIV ($p<0.01$); the densities of outgrowth were not increased by longer culture periods. It is interesting that the length of outgrowth did not increase linearly with the length of culture, i.e. the mean length of outgrowth increased more between 2DIV and 3DIV than during the first two DIV. It may take some time for outgrowth to become established.

Slices of cortex collected from E16 fetuses were also co-cultured with LGN explants, and outgrowth was observed in 95% of cases. The density of outgrowth was significantly higher than that observed when LGN explants were cultured alone ($p<0.01$), although it was on average about one fifth of that seen with postnatal cortex and there were no neurite fascicles. As before, outgrowth

occurred both towards and away from the cortical explants, and was about 70% as long as the outgrowth from LGN explants cultured with P2 occipital cortex.

4.3.2 Size of explants

The possibility that a relationship exists between the size of the target explant and its effectiveness in initiating outgrowth from an LGN explant was examined. For each combination of LGN explant and target tissue listed in Table 1, graphs were plotted of both the length and the density of outgrowth from the LGN explant against the size of the target explant. The ranges of size for each of the various types of target explant, including E16 cortex, were all similar and narrow. In no experimental group was there a significant correlation (tested with a regression analysis) between the size of the target explant and the amount of outgrowth from the LGN explant. Thus, within the ranges used here, the sizes of the explants were not critical in determining their effectiveness in stimulating LGN explants, and the effectiveness of different types of tissue could not be accounted for by differences in their sizes.

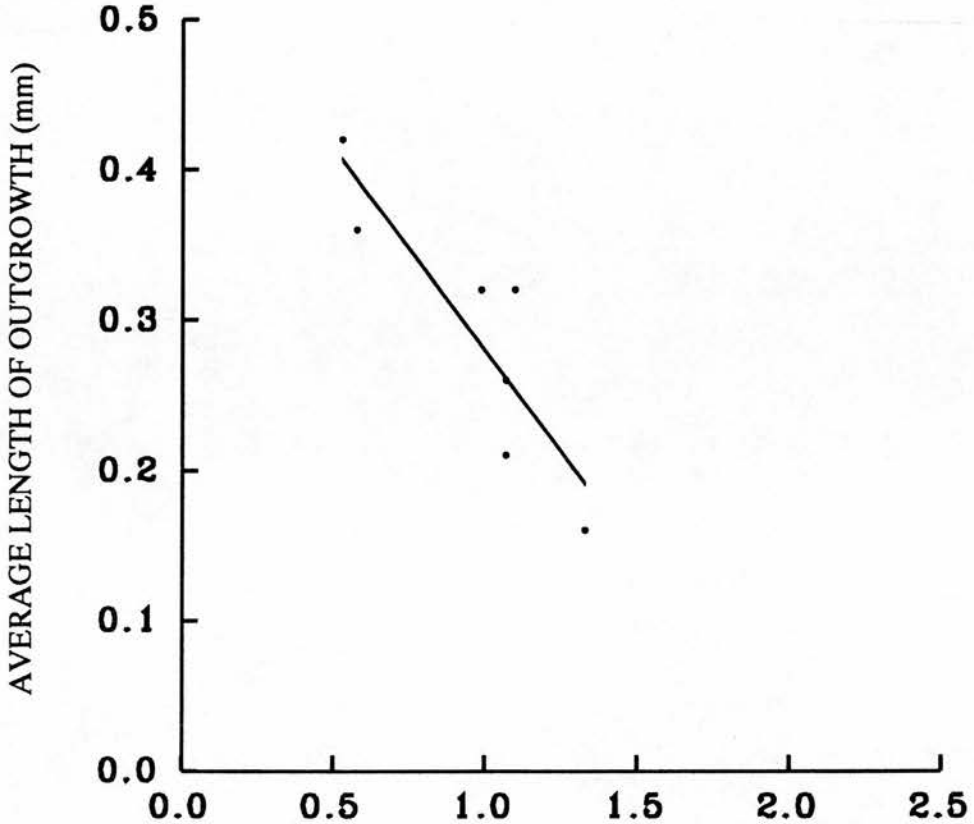
4.3.3 Distance between the explants

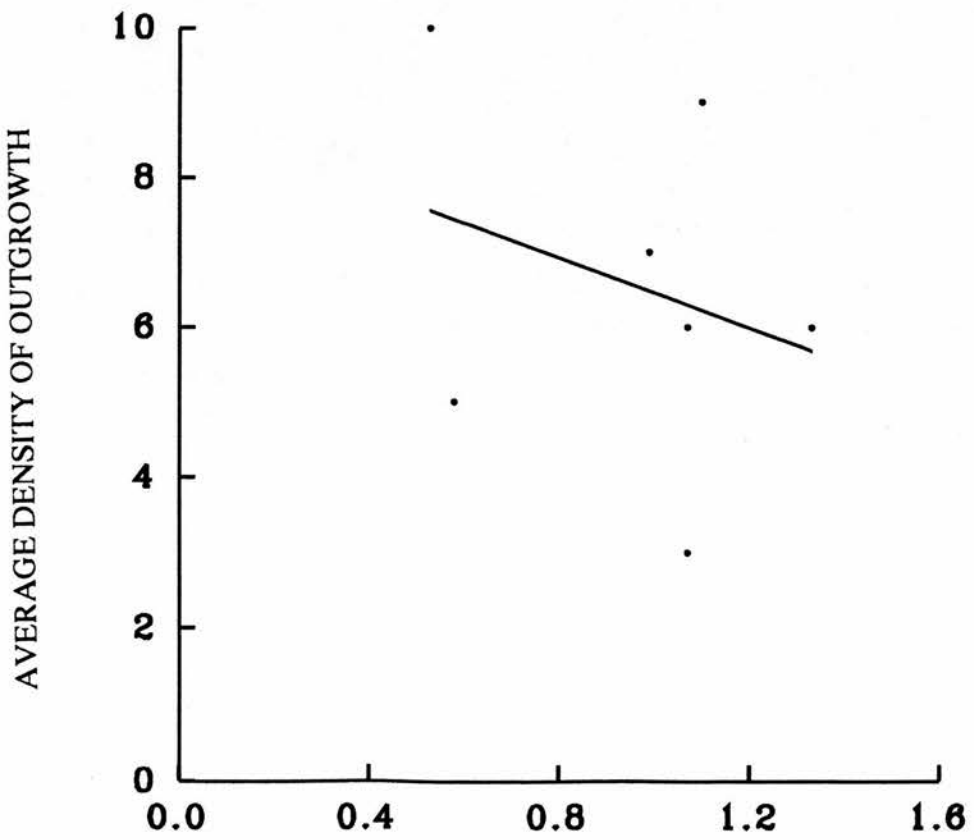
For all the experimental groups listed in Table 1 the explants were placed on the filters separated by a similar range of distances. The spreading of explants of medulla and liver was very limited, and this accounted for the distances separating their edges remaining at the upper end of the range (Table 1). For every combination of LGN explant and co-cultured tissue, I looked for a correlation between the distance separating the explants and the length and density of outgrowth produced from the LGN explant (regression analysis). In the vast majority of cases there was no significant correlation. I did not anticipate such correlations, and I conclude that differences in outgrowth with

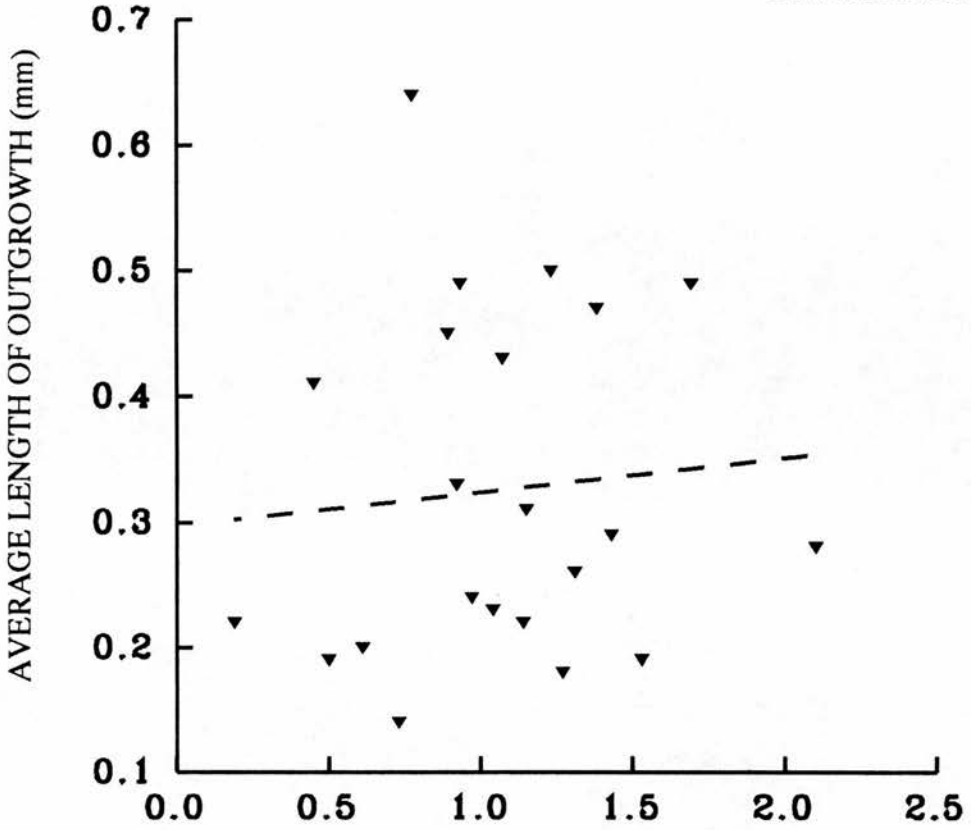
different tissues could not be accounted for by differences in separation between explants.

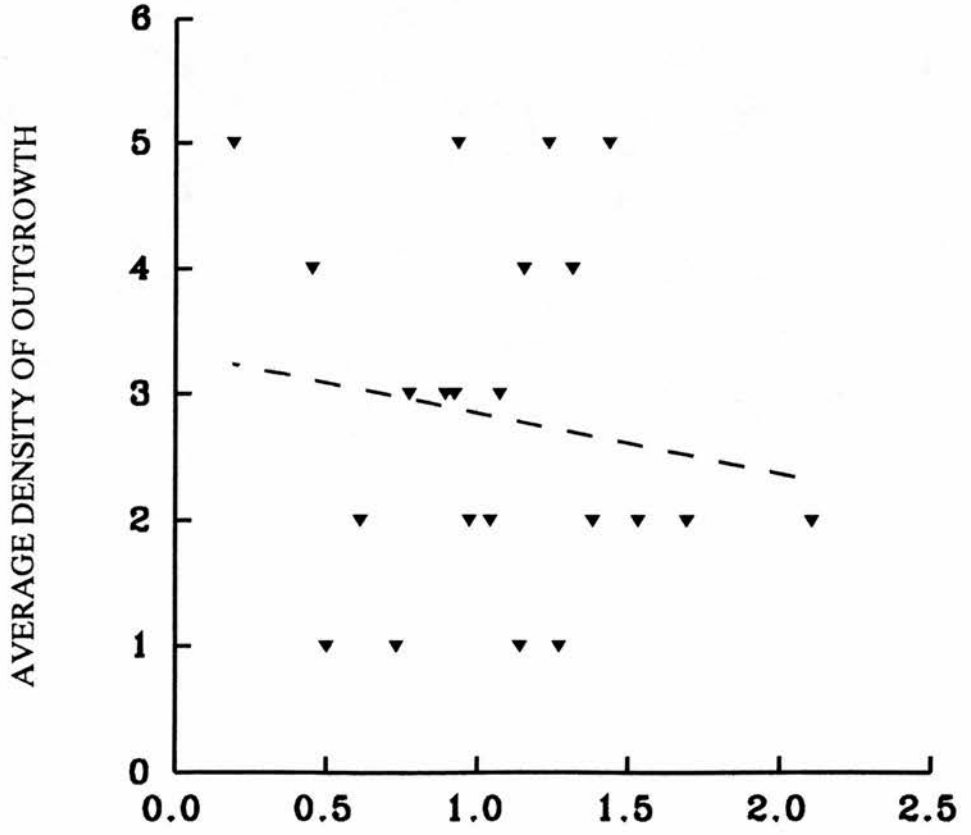
There was only one exception: after 2DIV, the length of outgrowth away from (but not that towards) explants of P2 occipital cortex were negatively correlated with the distance separating them (Fig. 9a). In Fig.9c and d, the regression lines for the data from cultures with frontal cortex are shown (broken lines). Regarding length, the data from cultures with occipital cortex are grouped around the regression line. Across the range of separations used here, the densities of outgrowth stimulated by occipital cortex were not significant.

Fig.9 Graphs plot (a) the length and (b) the density of outgrowth from LGN explants against their distance from co-cultured P2 occipital cortex and (c) the length and (d) the density of outgrowth from LGN explants co-cultured with P2 frontal cortex for 2DIV; outgrowth is on the side away from the cortical explant. Regression lines were calculated and are plotted. The only experimental group in which significant correlations between the amount of outgrowth and explant separation is highlighted in (a) with $p<0.001$.


AVERAGE LENGTH OF OUTGROWTH (mm)
0.5
0.4
0.3
0.2
0.1
0.0
0.0
0.5
1.0
1.5
2.0
2.5


AVERAGE DENSITY OF OUTGROWTH
10
8
6
4
2
0
0.0
0.4
0.8
1.2
1.6
AVERAGE DISTANCE BETWEEN THE EXPLANTS (mm)


AVERAGE LENGTH OF OUTGROWTH (mm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.5
1.0
1.5
2.0
2.5


AVERAGE DENSITY OF OUTGROWTH
6
5
4
3
2
1
0
0.0
0.5
1.0
1.5
2.0
2.5
AVERAGE DISTANCE BETWEEN THE EXPLANTS (mm)

4.3.4 Outgrowth from LGN explants is neuronal

Although it seemed likely that the outgrowth described in this paper was axonal, previous studies have shown that, under phase-contrast, glial cell outgrowth can appear remarkably similar to neurite outgrowth (Torran-Allerand, 1990). We confirmed that the outgrowth from LGN explants was axonal. Figure 10 (a and b) shows that the outgrowth was labelled with antineurofilament antibody.

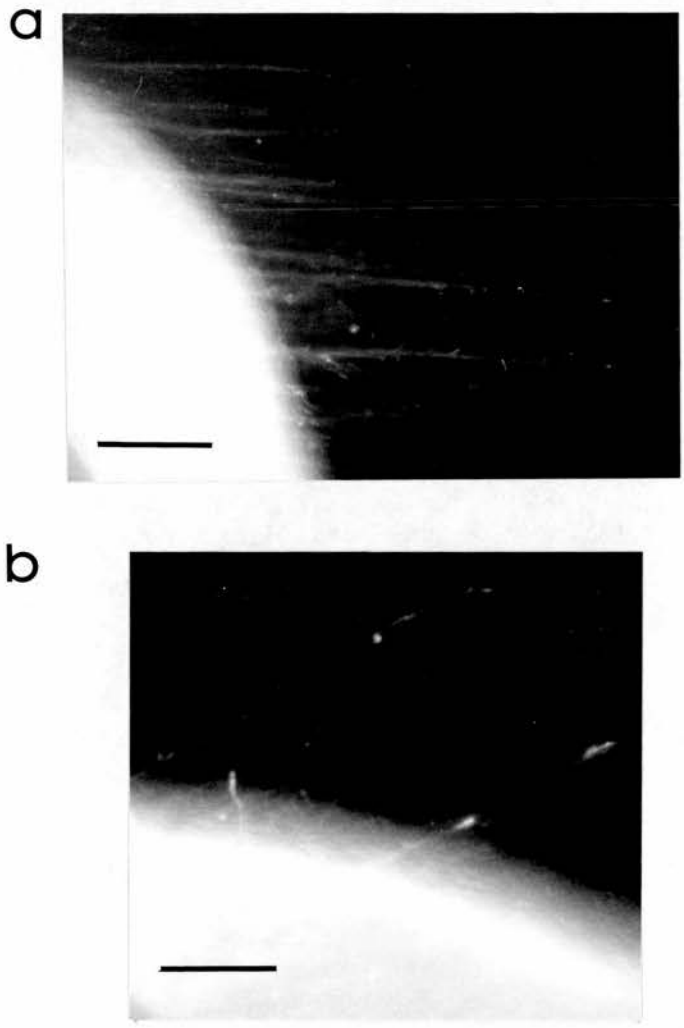


Fig.10 Fluorescence photomicrographs of neurites from LGN explants co-cultured with occipital cortex, labelled with antineurofilament antibody. In (a), fascicles of labelled neurites are seen emerging from the intensely labelled LGN explant. (b) Labelled neurites from a region where outgrowth was sparser; the collagen grooves can be seen faintly. Scale bars: a, 100um; b, 50um.

4.4 DISCUSSION

In recent years, several groups have begun using organotypic explant cultures to study the development of connections within the visual system. Previous studies were concerned mainly with ingrowth and target recognition by axons of various structures such as the LGN and the visual cortex (Yamamoto et al., 1989 and 1992; Blakemore and Molnar, 1990; Bolz et al., 1990 and 1992; Molnar and Blakemore, 1991; Toyama et al., 1991). The aim of our study was to use in vitro methods to search for possible long-range, chemically mediated interactions between the visual cortex and the LGN that may promote geniculate outgrowth and survival in vivo. We chose to use explants rather than dissociated cultures, since they preserve their organotypic organisation in vitro (Gahwiler, 1988; Yamamoto et al., 1989 and 1992; Bolz et al., 1990 and 1992; Molnar and Blakemore, 1991) and so are more likely to retain the potential for mimicking in vivo interactions.

We found that when LGN explants were cultured alone in chemically defined serum-free culture medium, there was either relatively little or no neurite outgrowth. However, when LGN explants were co-cultured (in the same type of medium) near to a slice of occipital cortex, they produced profuse outgrowth. The density of outgrowth increased up to 10-fold, and thick axon fascicles formed.

It is possible that the LGN explants, with or without the influence of other tissues, would have grown much more had I included serum in the culture medium, as others have done (Gotz et al., 1991; Bolz et al., 1992; Yamamoto et al., 1992). We did not include serum since its constituents are incompletely defined and certainly include fibronectin, vitronectin, chondronectin, fibrinogen, other adhesive molecules and a range of growth factors (Grinnell, 1978; Yamada, 1983; Hynes, 1985). Furthermore, the contents of serum vary from

batch to batch. We did not want the interactions in the co-cultures to be masked by the effects of serum. The serum-free conditions I used were not so harsh as to kill the cells in the LGN explants grown alone.

When frontal cortex and cerebellum were substituted for occipital cortex, the amount of outgrowth from the LGN explants was greater than from LGN explants grown alone, but less than that observed with occipital cortex. Medulla did not have a significant effect and liver had no effect at all. Thus, there was relative specificity, with the occipital cortex (the in vivo target of geniculate axons) having the greatest influence on the LGN explants. This makes it is extremely unlikely that the promotion of outgrowth was the result of a non-specific enrichment of the culture medium by cellular components, or very small quantities of serum that might have been carried with the explants (note that I also controlled for the size and separation of explants). The inability of the LGN to stimulate itself is further evidence against such a non-specific effect.

A close analysis of these results raises the possibility that the interaction between the occipital cortex and the LGN may be even more specific than suggested above. The major effect of altering the co-cultured tissue is on the density of outgrowth from an LGN explant. Thus, it could be that different sets of cells, each capable of growing to a similar length, are stimulated by the occipital cortex, frontal cortex or cerebellum. As stated earlier, the LGN explants are unlikely to include only LGN. We are confident that they contain mainly LGN, but almost certainly they include parts of other neighbouring thalamic nuclei. For example, the dorsomedial nucleus, which projects to the frontal cortex, and the ventrolateral nucleus, which is connected to the cerebellum, are close to the LGN. It is possible that different regions of the brain release different substances, each stimulating a specific thalamic nucleus.

Assessing this possibility will be difficult, since it is hard to obtain thalamic explants that include only a single nucleus, even in rats (Yamamoto et al., 1992). Alternatively, it could be that all cortical regions and cerebellum release substances that stimulate the LGN (and perhaps other thalamic nuclei as well). The reasons for the weaker effects of areas other than occipital cortex may be that they release different factors that have less influence on the LGN, or that they release the same factors but in lower quantities, at least at P2 and perhaps throughout development.

In this study, I took LGN explants at E16, since this is the time around which outgrowth from the LGN begins in vivo (Lund and Mustari, 1977; Molnar and Blakemore 1990; Catalano et al., 1991; De Carlos and O'Leary, 1992; our unpublished observations - see chapter 3). As I wished to minimise the damage inflicted on geniculate neurones by denervation during their preparation for culture (Bolz et al., 1992). For most of this work, I used slices of cortex or other tissue from early postnatal animals, so that I could be sure that the appropriate target cells were present. However, I did test whether growth-promoting factors can be released from cortex at the time that geniculocortical growth begins in vivo. Slices of cortex were taken at E16 (they were from the caudal pole of the brain, although I was not sure that they contained the exactly equivalent region of occipital cortex as in the postnatal explants, in view of the small size and immature form of the brain). The E16 cortical slices stimulated significant outgrowth from E16 LGN explants, although not as much as when LGN explants were cultured with neonatal occipital cortex. This could have been because, at E16, only about half of the cortical layers are present and few of the cells destined for layer 4 (a potential source of a factor affecting the LGN) are in the cortex (Lund and Mustari, 1977; our unpublished observations). These results suggest that, at the time when geniculocortical fibres start to grow

in vivo, they may be stimulated by the cortex. Thus, the factors suggested by the results of this study may both initiate and maintain or accelerate geniculate outgrowth in vivo.

It appeared that the grooves in the collagen filter provided mechanical guidance for axons emerging from the LGN explants. Studies in other systems have demonstrated that neurites orientate themselves to grow along large grooves about 5um wide, although not along fine grooves 130nm wide or less (Clarke et al., 1990 and 1991). Ebendal (1976) has shown that neurites will align themselves to grooves on collagen-coated coverslips. Whether or not the ability of collagen grooves to influence growth from LGN explants in vitro has relevance in vivo is an open question, since it is unclear if structures with similar properties to these grooves exist in vivo.

In conclusion, I suggest that the embryonic LGN are stimulated to grow geniculocortical axons by diffusible factors releasd from their target cells in the occipital cortex . It is possible that other regions of the developing brain also release the same factors. These factors may continue to enhance the elongation of geniculocortical axons as they grow to and within their targets. I believe that the guidance of geniculocortical axons to their specific targets is likely to depend on other mechanisms.

CHAPTER 5

INVESTIGATION OF THE FACTOR PRODUCED BY THE VISUAL CORTEX

5.1 INTRODUCTION

The results in the previous chapter suggest the presence of a factor involved in the development of connections between the LGN and the visual cortex, but they give no indication of the nature of the factor. The aims of the experiments described in this chapter were firstly to confirm that factors were released by the visual cortex and that they are freely diffusible in the culture medium.

Secondly, I investigated the effect this factor was having on the LGN, that is was it having a trophic effect? (For more information on this class of growth factor and its mode of action see Introduction, section 1.6).

Finally, I attempted to mimic the effect the occipital cortical explant was having on the LGN by adding nerve growth factor (NGF) to the culture medium. The study by Domenici et al. (1991), where NGF was observed to have a role in the plasticity of the visual cortex, led me to hypothesise that NGF was a candidate for the factor released by the occipital cortex.

In summary, a series of experiments were carried out to investigate the nature and action of the interaction detailed in chapter 4, which seems to be involved in the formation of the geniculocortical pathway.

5.2 MATERIALS AND METHODS

5.2.1 Animals and surgery

Tissue was collected following the procedure similar to that described in chapter 4, section 4.2.1, Animals and Surgery.

5.2.2 Identification of the LGN

The LGN explants were identified and collected as described in chapter 4, section 4.2.2, Identification of the LGN.

5.2.3 Culture methods

The tissue was cultured following the protocol described in chapter 4, section 4.2.3, Culture Methods.

5.5.4 Conditioned medium experiments

Four to six 350um-thick slices of occipital cortex from P3 mice were cultured alone in serum-free medium on the collagen-coated filters, following the procedures described previously. The period of culture was termed the conditioning period, and lasted for either 2 days (2-day conditioned medium: 2DCM) or 4 days (4DCM).

On the day the conditioned medium was decanted, LGN explants were dissected from E16 fetuses and were placed on collagen filters that had been pre-incubated for 2 hours with unconditioned culture medium. Once these explants were in place, the medium was replaced with the conditioned medium; 21 cultures were carried out with 2DCM, and 31 with 4DCM. The cultures were incubated at 37^{o}C and 5% CO_2 for 3 DIV and then fixed with 4% para-formaldehyde in phosphate buffer. In 17 control experiments, LGN explants were cultured with chemically defined serum-free medium that had been incubated at 37^{o}C and 5% CO_2, with no tissue added, for 2 days simultaneous with the conditioning period.

5.2.5 Analysis

Quantification was carried out as before (chapter 4, section 4.2.4, Analysis). In this chapter, however, mean values for each explant were calculated using all six measurements, three on each side of the explant.

5.2.6 Light and electron microscopy

LGN explants that had been cultured either with occipital cortex or alone (in unconditioned medium) were fixed in 2.5% gluteraldehyde, 2% paraformaldehyde and 3% sucrose in phosphate buffer, and then embedded in blocks of acrylic resin (medium grade). For electron microscopy, sections were cut with an ultramicrotome, mounted on copper grids, and stained with uranyl acetate and lead citrate. For semithin sections, 0.5-1um sections were cut and mounted onto glass slides, stained with toluidine blue, coverslipped and observed using a compound microscope. Data were obtained from 3 LGN explants cultured with and 3 LGN explants cultured without occipital cortex. For each of 8-13 sections taken from each explant, the numbers of healthy and pyknotic cells (identified as described in Results) in 8-10 randomly positioned areas ($0.04mm^2$) were counted. The mean densities of healthy and pyknotic cells and the ratios between them were then calculated and compared statistically (comparison of means).

5.2.7 Addition of NGF to the culture medium

Collagen filters were pre-incubated for 2 hours in control medium at 37^o and 5% CO_2, as described previously (section 4.2.3, Culture Methods). Prior to the addition of LGN explants to the filter, the previously incubated culture medium was removed and replaced with medium supplemented with nerve growth factor (NGF). Three concentrations of NGF were added: 10ng/ml, 20ng/ml and

40ng/ml. For the remainder of the experiment, the protocol discussed in section 4.2.3, Culture Methods was followed.

5.3 RESULTS

5.3.1 Conditioned medium

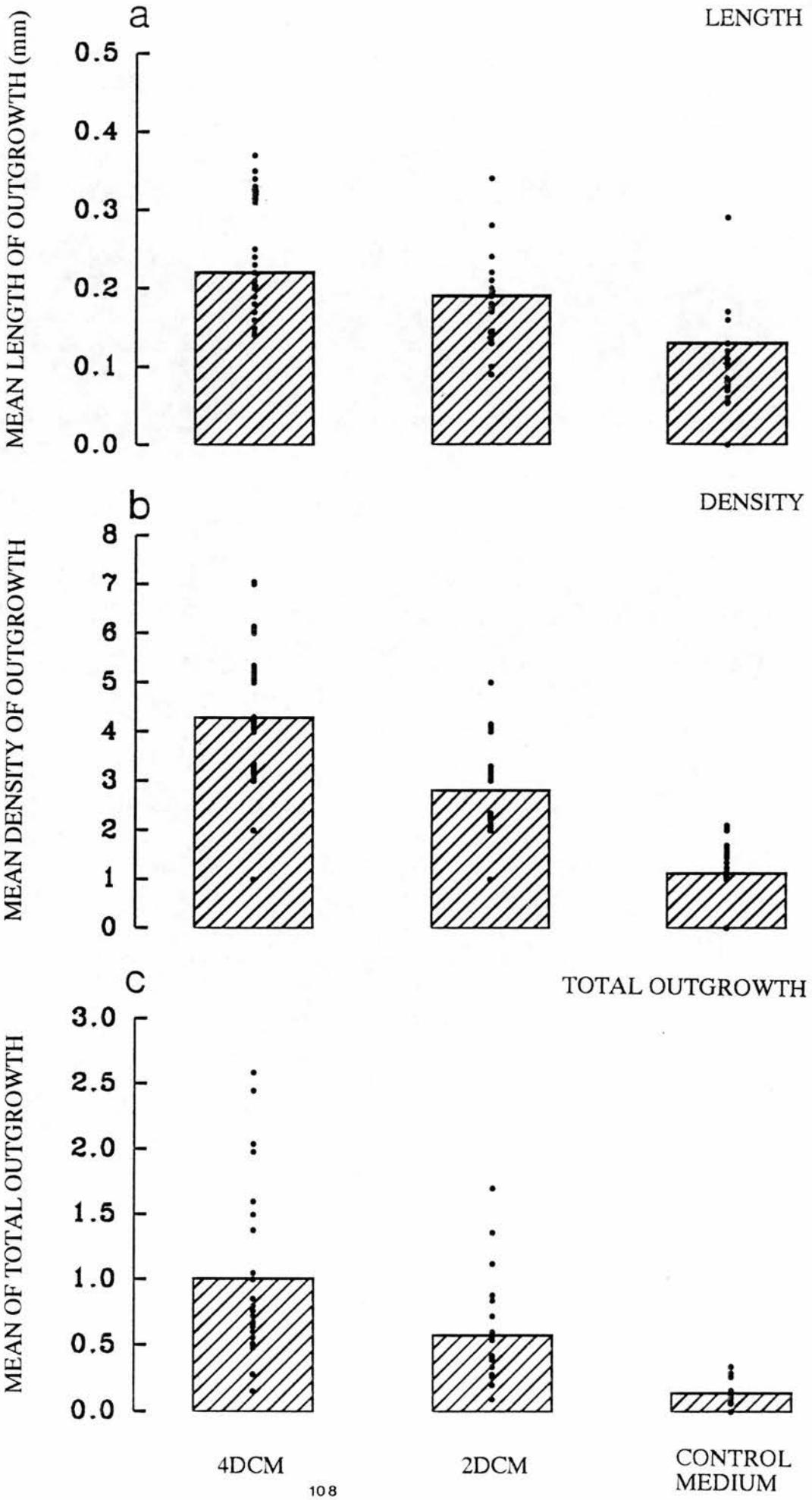
To investigate further the possibility that the occipital cortex produces a soluble and diffusible factor that stimulates the LGN, LGN explants were cultured for 3 DIV in medium that had been pre-conditioned with 4-6 slices of P3 occipital cortex for 2 days (2DCM) or 4 days (4DCM) (see Table 1).

Explants	Medium	DIV	No. of Expts
E16 LGN	4DCM	3	31
E16 LGN	2DCM	3	21
E16 LGN	CONTROL	3	17

Table 1. Summary of experimental repeats with medium conditioned by visual cortex. Abbreviations, DIV: days in vitro; expts: experiments; 4/2DCM: 4/2 day conditioned medium.

The results are shown in Fig. 1. In these cultures, the majority of the outgrowth from the LGN explants followed the grooves on the collagen filters. Quantification was carried out as before, but mean values for each explant were calculated using all six measurements (three on each side of the LGN explant). The histogram in Fig. 1a shows the lengths of outgrowth from LGN explants cultured with control medium, 2DCM or 4DCM. The mean length of outgrowth in 4DCM was only 14% greater than in 2DCM (statistically insignificant), but was 41% greater than that observed in control medium. The differences between the length of growth in control medium and in either 2DCM or 4DCM were significant ($p<0.01$). Fig.1b compares the density of outgrowth from the LGN explants cultured with 4DCM, 2DCM and control medium. The average density of outgrowth was highest in 4DCM, being 34% higher than in 2DCM, and 74% higher than in control medium; all these differences were significant ($p<0.01$). In Fig.1c, data on total outgrowth (density of outgrowth x length of outgrowth) are presented. Again the total outgrowth observed was greatest with 4DCM, was less in 2DCM, and least in control medium; these differences were all significant ($p<0.01$).

Fig. 1 Histograms show (a) the mean lengths of outgrowth, (b) the mean densities of outgrowth and (c) the means for total outgrowth (length x density) from LGN explants cultured in medium conditioned with P3 occipital cortex for 2 or 4 days (2 or 4DCM). Control experiments were carried out with culture medium pre-incubated for 2 days, simultaneous with the conditioning period. Data from both sides of the explants are combined; otherwise, graphs are plotted as in Figs. 7 and 8, chapter 4.


a
LENGTH
MEAN LENGTH OF OUTGROWTH (mm)
0.5
0.4
0.3
0.2
0.1
0.0
b
DENSITY
MEAN DENSITY OF OUTGROWTH
8
7
6
5
4
3
2
1
0
c
TOTAL OUTGROWTH
MEAN OF TOTAL OUTGROWTH
3.0
2.5
2.0
1.5
1.0
0.5
0.0
4DCM
2DCM
CONTROL
MEDIUM

In general, the results from these conditioned medium experiments followed the same pattern as those from the co-culture experiments. The effect of using conditioned medium rather than control medium was more pronounced when the density of outgrowth, rather than its length, was considered. The average densities of the outgrowth after 3DIV in 2DCM and 4DCM were similar to those from LGN explants co-cultured with P3 occipital cortex for 3 DIV (compare Fig. 1b and Fig. 8b of section 4.3.1, Outgrowth from the LGN explants cultured either with or without target tissues). The lengths of outgrowth in conditioned medium were lower than in comparable co-culture experiments (compare Fig. 1a and Fig. 8a of section 4.3.1, Outgrowth from LGN explants cultured either with or without target tissue). In conditioned medium, 100% of LGN explants produced outgrowth; in control medium preincubated for 2 days, 94% of explants grew after 3 DIV. In previous cultures, where the collagen filters and culture medium were preincubated at $37^{o}C$ and 5% CO_2 for 2 hours, the proportion of LGN explants cultured alone that produced outgrowth after 2DIV was only 14% (see above). Thus, it appears that preincubation of the culture medium for 2 days and/or culture for 3 DIV can reduce the failure rate of LGN explants grown alone. However, the outgrowth that did occur in the controls for the conditioned medium experiments (Fig. 1) was neither significantly longer nor denser than that from LGN explants cultured alone in the co-culture experiments (Fig. 7 section 4.3.1, Outgrowth from LGN expalnts cultured either with or without target tissue).

5.3.2 Cellular content of LGN explants

The cellular content of E16 LGN explants cultured either alone or with occipital cortex was examined in semi-thin sections using the light microscope (Fig. 2). I differentiated between healthy cells and those that were dead or dying (all termed "pyknotic" cells); I could not differentiate between dead and dying cells. Pyknotic cells were identified by their small, dark nucleus, which was often fragmented into several smaller pieces; the cytoplasm of these cells was often absent (Ferrer et al., 1992). In LGN explants cultured alone, the mean density of healthy cells was 3.6×10^3 mm^{-2} and the mean density of pyknotic cells was 1.0×10^3 mm^{-2}. In LGN explants cultured with occipital cortex, the mean density of healthy cells was 3.0×10^3 mm^{-2} and the mean density of pyknotic cells was 1.0×10^3 mm^{-2} (neither value was significantly altered). There was very little variation in the values between individual LGN explants. Pooling data from LGN explants cultured alone, 78% (n=16,794) of the cells appeared healthy, and 22% (n=4,737) of the cells appeared pyknotic. For the three LGN explants cultured with occipital cortex, 75% (n=15,546) of the cells were healthy and 25% (n=5,179) of cells were pyknotic. Therefore, the presence or absence of occipital cortex did not influence the survival of cells in the E16 LGN explants.

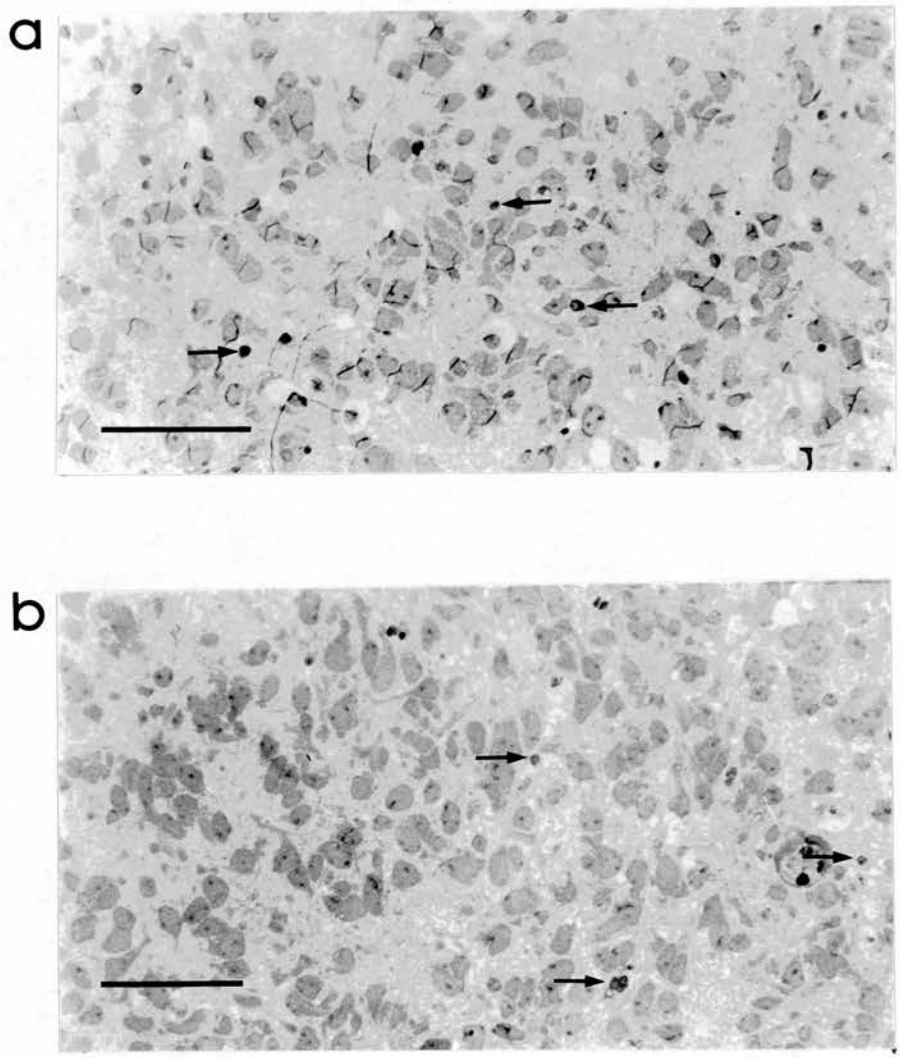


Fig. 2 Photomicrographs of semi-thin sections of LGN explants that were co-cultured (a) with occipital cortex, or (b) alone. In both cases, the majority of cells appeared healthy. The arrows indicate examples of pyknotic cells. In (a), the fine dark lines are artefacts introduced by slight wrinkles in the sections. Scale bars, (a) 28um and (b)45um.

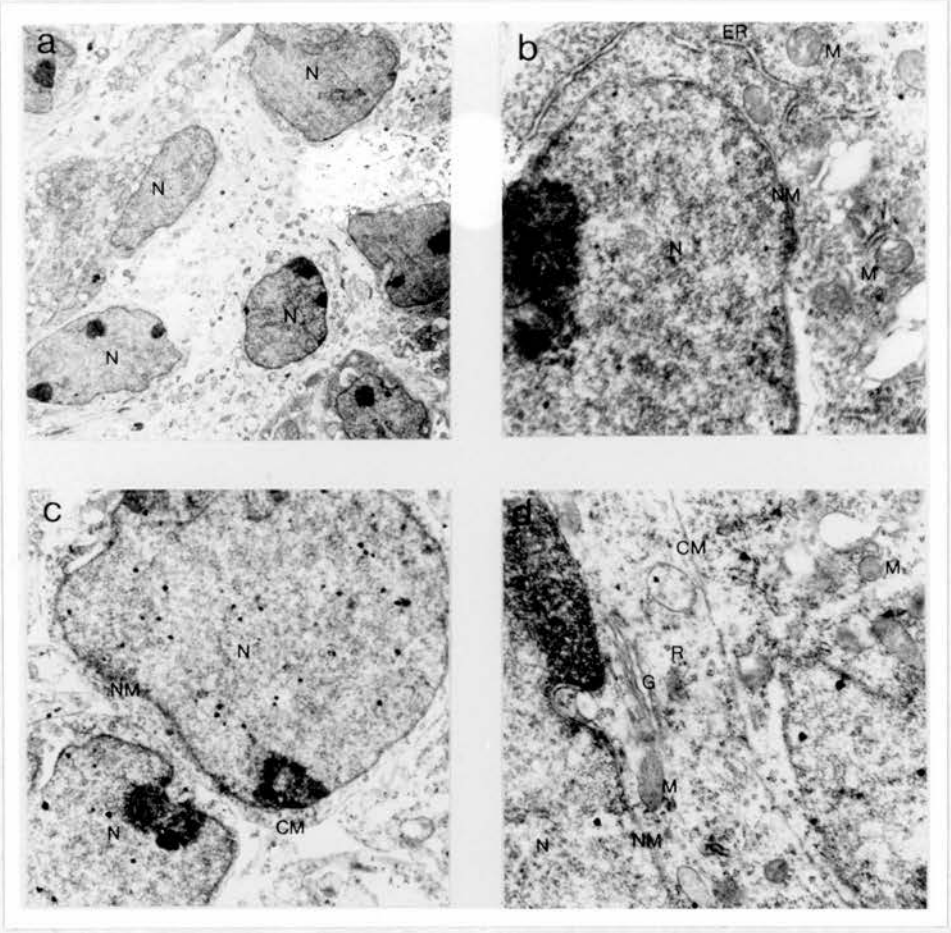


Fig. 3 Electron micrographs of sections of LGN explants that were cultured with occipital cortex (a,b) or alone (c,d). (a) A low-power overview after culture with occipital cortex, showing several healthy nuclei (x4,500). (b) A higher-power view of an LGN explant cultured with cortex, showing a single nucleus with its nuclear membrane; some inner mitochondrial membranes are slightly damaged, although others have been preserved (x30,000). (c) A low-power view of an LGN explant that had been cultured alone. The nuclei and nuclear membranes are seen (x15,000). (d) A higher-power view of an LGN explant cultured alone. As in (b), the inner mitochondrial membranes are in the main healthy, although some show signs of deterioration (x30,000). The abbreviations are: N, nucleus; NM, nuclear membrane; CM, cell membrane; ER, endoplasmic reticulum; G, Golgi; R, ribosomes; M, mitochondria.

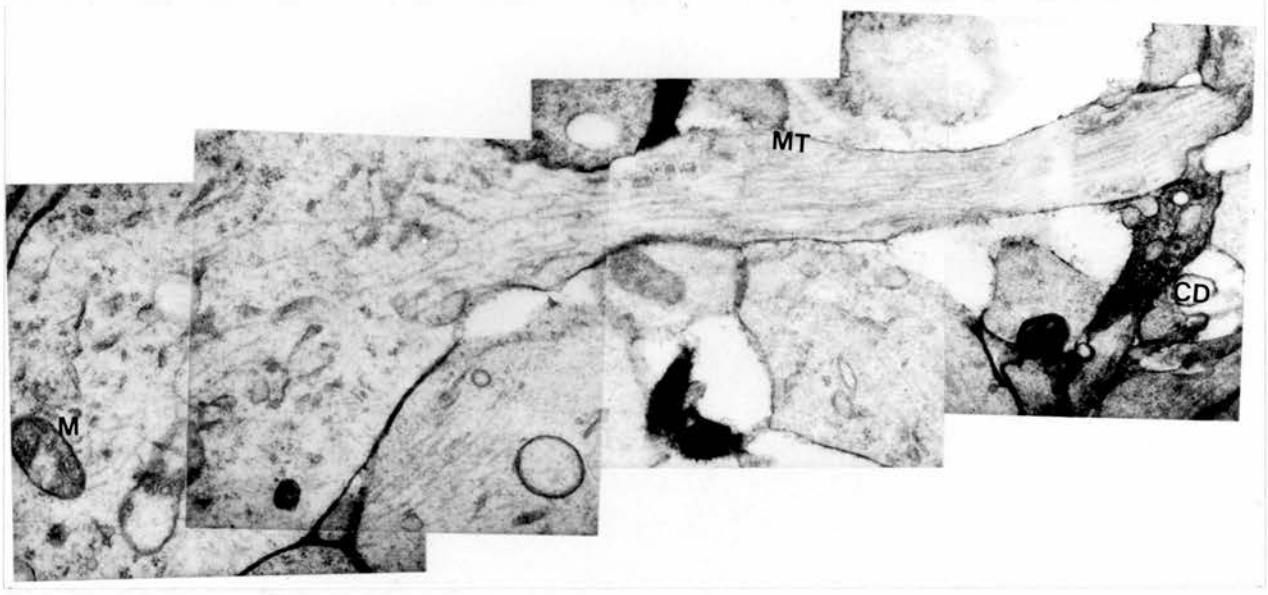


Fig. 4 Electronmicrograph of a section of an LGN explant cultured alone for 2DIV, showing a dendrite. Microtubules are observed running the entire length of the dendrite. Like the organelles highlighted in Fig. 3 the mitochondrial inner membranes appear to be slightly damaged. Some cellular debris can also be observed. The abbreviations are: MT, microtubules; M, mitochondria; CD, cellular debris.

Further evidence in support of this conclusion was obtained by examining the ultrastructure of LGN explants. Figures 3 and 4 show electron micrographs of sections of LGN explants cultured either with (Fig. 3a and b) or without (Fig. 3c, d and 4) occipital cortex. In both experimental groups, explants contained many intact cells with healthy-looking nuclei. There were some signs of damage, although they were as frequent in explants cultured with cortex as in those cultured without. For example, some of the mitochondrial inner membranes were slightly damaged (Fig. 3b and d). Although the damage that I observed may indicate that the tissue was beginning to deteriorate in vitro, it is possible that at least some resulted from the fixation of the tissue. I concluded that the cultured LGN explants were largely healthy, and that unhealthy features (which have been described previously in organotypic explant cultures, see Wolburg and Bolz, 1991) appeared with similar frequency whether or not occipital cortex was present in the cultures.

5.3.3 Effects of NGF on outgrowth from the LGN explants

The results are summarised in Table 2. When cultured with NGF, little or no outgrowth was observed from LGN explants. The outgrowth that did occur consisted of a small number of single neurites. Axon fascicles were never seen, unlike the outgrowth observed from the LGN explants cultured with slices of occipital cortex.

Explants	DIV	Conc.NGF	No. of Expts.	Outgrowth +	-
E16 LGN	3	10ng/ml	30	20	10
E16 LGN	3	20ng/ml	30	22	8
E16 LGN	3	40ng/ml	30	25	5

Table 2. Summary of the total numbers of experiments, and the numbers of LGN explants that produced outgrowth (+) or not (-) in response to the presence of NGF in the medium. Abbreviations, DIV: days in vitro; Conc. NGF: concentration of nerve growth factor; expts: experiments.

As the concentration of NGF added to the cultured LGN explants increased, the proportion of explants that produced outgrowth increased (Table 2). When 10ng/ml of NGF was added to the culture medium, 10 out of 30 explants failed to produce any outgrowth (not even a single neurite was observed growing from these explants). With 20ng/ml of NGF, the number of LGN explants that failed to produce outgrowth dropped to 8 out of 30 and when 40ng/ml of NGF was added this fell again to 5 out of 30. However, despite this

increase in the number of LGN explants which produced outgrowth, the length and density of outgrowth did not increase. Even in the presence of the highest concentrations (40ng/ml) of NGF, the outgrowth from the LGN explants still consisted of single neurites emerging randomly from the explant.

It is apparent from these results that LGN explants do not respond significantly to NGF. A marginal effect cannot be ruled out altogether. It is, unlikely that NGF is the factor produced by the occipital cortex that is responsible for the interactions that I have identified.

5.4 DISCUSSION

When LGN explants were cultured alone in medium that had been pre-conditioned with slices of occipital cortex, the density of outgrowth increased from control levels to levels comparable with those observed when LGN explants were cultured with occipital cortex. The amount of outgrowth was significantly greater when the conditioning period was lengthened. Thus, I conclude that the occipital cortex can secrete a diffusible substance(s) that stimulate(s) the outgrowth of neurites from LGN explants.

Most cells in the LGN explants remained alive during the culture period, whether or not the occipital cortex was present. Thus, trophic factors released by the occipital cortex did not enhance the survival of cells in these explants. It would be reasonable to suppose that the early embryonic LGN is not dependent on its target for survival since, in vivo, the LGN is born before most of the cortex and certainly before layer 4 cells. The results of Cunningham et al. (1987) have suggested that a factor produced by the postnatal visual cortex is required for the survival of the postnatal LGN. It is possible that growth factors influence the outgrowth and morphology of geniculocortical axons before they

develop a trophic role, as occurs in other systems (Wright et al., 1992).

It was thought that NGF may be the factor produced by the occipital cortex which stimulates outgrowth from the LGN explants. However, when this was added to the culture medium little or no outgrowth was observed from the LGN explants and the outgrowth that was observed was no greater than that observed from LGN explants cultured in control culture medium. The concentration of NGF used by other workers in most similar experiments is 20ng/ml. For example, Hamburger and Yip (1984) in their study of neuronal cell death in the spinal ganglia of the chick embryo. In the mouse the highest concentration of NGF in vivo is associated with the sub maxillary gland which is the most highly innervated target-field. On E13 NGF levels peak at around 2.0pg, that is 80pg/mg of protein (Davies et al., 1987). Therefore, even at double the concentration used normally NGF has no effect on the LGN explants. Similarly, Hisanaga and Sharp (1990) observed that NGF had no effect on the survival of dissociated thalamic cells in culture. It is unlikely, therefore, that NGF is the molecule produced by the occipital cortex that is responsible for provoking geniculate outgrowth in vitro.

In conclusion, I suggest that the occipital cortex releases diffusible factors that stimulate outgrowth from the embryonic LGN. The survival of the early embryonic LGN appears not depend on these factors, although the survival of the postnatal LGN may do. It is unlikely that NGF is the factor produced by the occipital cortex.

CHAPTER 6

SUMMARIES AND CONCLUSIONS

6.1 SUMMARY OF MAIN POINTS

1. The migration of cells destined for cortical layer 4, from the ventricular surface into the cortical plate, was investigated. Cells were labelled with bromodeoxyuridine (BrdU) on E14 and E15 and their migration monitored on subsequent days, both before and after birth. It was noted that the migration of layer 4 cells into the cortical plate is complete by the day of birth. They begin to take up their final positions around P3, however migration is still occuring until after P7.

2. The birth of the LGN was also investigated, using BrdU to birth date the cells. I observed that the generation of geniculate cells was complete by E14. No labelled cells were observed on either E15 or E16.

3. I also investigated the in vivo development of the geniculocortical pathway. DiI was used to label the geniculate axons as they progress to the visual cortex. It was shown that on E17 geniculate axons have reached the cortical plate.

4. The development of the geniculocortical pathway was also investigated using in vitro techniques. LGN explants were either cultured alone or with slices of occipital cortex, frontal cortex, cerebellum, medulla or liver. It was found little or no outgrowth occured from the LGN explants when cultured alone. However, when occipital cortex was added neurites grew from the LGN. Outgrowth was also observed from the LGN explants when they were co-cultured with frontal cortex, cerebellum and medulla, although significantly less than was observed with occipital cortex. There was no outgrowth from the LGN explants when liver was co-cultured. The main effect on outgrowth from

LGN explants was on the density of outgrowth, the length of outgrowth varied little. From this I concluded that the occipital cortex produced a factor which the LGN explants responded to by producing neurite outgrowth.

5. The nature of this factor was next investigated. Conditioned medium experiments demonstrated that the factor produced by occipital cortex was diffusible.

6. The factor was found not to be required by E16 LGN explants for the survival of the majority of cells within them, making it unlikely that it was having a trophic effect on LGN at this stage in development.

7. NGF was added to control culture medium to see if it could mimic the effects observed when LGN explants were co-cultured with occipital cortex. I found that NGF had virtually no effect on outgrowth from the LGN, making it unlikely that NGF was the factor produced by the occipital cortex.

6.2 OVERVIEW OF THESIS

This study employed similar techniques to those used by Yamamoto et al. (1989 and 1992), Bolz et al. (1990 and 1992), Molnar and Blakemore (1991) and Toyama et al. (1991). However unlike the aforementioned studies I was interested in the outgrowth of neurites from LGN explants rather than target-recognition in the cortex. The period of culture was deliberately short ensuring that the geniculate outgrowth did not contact its co-cultured target explant. The main effect observed when comparing outgrowth in cultures with occipital cortex and that present when LGN was cultured on its own, was on the density

of outgrowth; the length of outgrowth varied little under the various culture conditions. The increase in density observed with cortical tissue implies that the factor produced by the cortex is responsible for neuron activation. That is, the factor may be responsible for stimulating a particular set of cells to produce outgrowth.

I also observe that frontal cortex produces a significant increase in outgrowth when compared to that seen when LGN explants were cultured alone. I hypothesised that this may be due to the frontal cortex producing (i) a different factor to that produced by occipital cortex, but which LGN explants can also respond to or (ii) the same factor as the occipital cortex but in reduced concentrations. I think that the latter is most likely to be the case. The complexity which would be involved in a system where each region of cortex would produce a novel factor would be great.

It is unlikely that area specificity, e.g. is the innervation of murine visual cortical area 17 as opposed to the innervation of either area 18a or 18b by a particular set of axons, is controlled by chemical factors. It is more likely that the subplate cells play a part in this, highlighted by Ghosh et al. (1990). (For more information on this study see section 1.5.4 Innervation of correct cortical areas).

The BrdU study discussed in chapter 2 showed that layer 4 cells undergo a surprisingly long period of migration which is not complete until after P7. The initial period of migration is very rapid, with labelled layer 4 cells observed in the cortical plate as early as E16. The migration of cells into the cortical plate is complete by the day of birth (E21/P0).

It is possible to hypothesise that the initial migration of cells is very rapid to ensure that these cells are present in the cortical plate when the geniculate axons arrive. Based on the observations discussed in chapter 3, and

those discussed in the introduction section 1.5.2 (Evidence disputing the waiting period), it is probable that in the mouse geniculate axons do not undergo a waiting period. This being the case cell migration may slow down because the area is now very crowded with geniculate fibres and the cells are under physical restrictions.

Migration apart, the results presented in chapter 2 clearly demonstrate that when slices of occipital cortex are cultured layer 4 cells are present in the cortical plate (even those cultures carried out with slices of E16 cortex). If the factor produced by the occipital cortex is responsible for inducing geniculate axons to grow from the LGN it is likely that it would be produced by their target cells, that is layer 4 cells. However, it could be argued that at this key stage in development the subplate cells are equally as important as layer 4 cells, and some groups may go as far as arguing that the subplate cells are more important. It would therefore be extremely interesting to investigate the presence and migration of the subplate cells in a study similar to the layer 4 birthdating study. Wood et al. (1992) highlighted the presence of a transient population of cells born on E12; these cells were reported to have completely disappeared in the adult animal. It would be interesting to know the fate of these cells and compare their presence with the production of the stimulating factor highlighted in chapter 4. It could be hypothesised that it is in fact the subplate cells that are producing the stimulating factor and not layer 4 cells.

One of the key problems with interpretation arose from the conditioned medium experiments discussed in chapter 5. Here control experiments, that is experiments were the LGN explants were cultured in defined medium which had been preincubated for 2 days in tandem with the conditioning period, showed an increase in the number of LGN explants which produced outgrowth. Only 14% of LGN explants produced outgrowth when cultured alone in

experiments were the culture medium was preincubated for 2 hours (experiments discussed in chapter 4). However, after a 2 day preincubation of culture medium this increased to 94%. It should be noted though that the outgrowth observed here was neither longer nor denser than that observed in the previous experiments.

These results are very confusing and I find it difficult to know what is happening in the conditioned medium experiments to cause such an increase in the numbers of LGN explants producing outgrowth. One simple explanation may be that the 2 day preincubation period ensures that the culture medium is properly gased and therefore, fully oxygenated. The 2 hour incubation period may not be long enough to achieve this. If this was the case however, one might ask why then do LGN explants cultured with occipital cortex produce outgrowth. It may be that the factor produced by the occipital cortical explant provides enough nutrients which will compensate for the lack of proper oxygenation and sustain the LGN explants until the medium is properly gased.

6.3 CONCLUSIONS

The occipital cortex produces a factor or factors which stimulates LGN explants to produce neurite outgrowth. Most of the experiments which resulted in the above conclusion were carried out using early postnatal cortical tissue. E16 cortex was also shown to stimulate significantly more outgrowth than was observed from LGN explants cultured alone. The first geniculate axons reach the visual cortex around E17. Therefore the factor produced by the occipital cortex is likely to have a physiological role in the development of the geniculocortical pathway.

I propose that early in development the visual cortex produces this factor. The

factor may be responsible for initiating outgrowth from the LGN. I speculate that the factor is involved in guiding thalamic fibres to the cortex (rather than to some other place). If this is the case subplate interactions maybe involved in the control of specific innervation patterns.

Early geniculate cells do not require the factor for survival, although experiments by Cunningham et al. (1987) show that postnatal geniculate cells do depend on the cortex for survival. It may be that the factor identified here has a dual role in development. Firstly, it may stimulate outgrowth from the LGN or direct the outgrowth to the visual cortex. Once the geniculate axons have invaded the correct area of cortex, and formed synapses with the appropriate cells (those of layer 4), the factor may then become involved in the maintenance of these synapses.

6.4 FUTURE WORK

We are now approaching a new and exciting period. This study is the foundation of a great number of experiments where both the identity and the role of the factor produced by the occipital cortex are being investigated.

1. I plan to carry out a series of experiments where I add identified growth factors to the LGN explants to see if I can mimic the outgrowth observed when occipital cortex is present (similar to the experiments carried out using NGF). The factors I intend to test in this way are brain derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and fibroblastic growth factor (FGF).

2. I intend to search for the expression of the trk receptors (the receptor family involved in binding the neurotrophin molecules, that is, NGF, BDNF and

NT3) in the LGN explants using in situ hybridisation techniques.

3. In tandem with these experiments I intend to carry out a biochemical investigation of the factor. Firstly, it is important to establish if the factor is a protein. This will be investigated by testing the heat sensitivity of the factor, and the effects proteases have on its activity. While doing this it would be wise to test the effects of polysaccharidases on the activity of the factor, to see if activity is dependent on sugars.

4. Western blots could be carried out on conditioned culture medium to compare the sizes of released factors to those of known growth promoting factors to see if it might already have been identified (this is if any are similar to known growth factors of the neurotrophin family).

If the factor is novel the next stage would be to isolate it. Firstly, the factor should be roughly isolated by size fractionation. It can then be purified using one of several techniques (i) ion-exchange chromatography, (ii) molecular exclusion chromatography or (iii) HPLC.

Once the purified molecule has been obtained antibodies can be generated. These could be used in an assay to block the activity of the factor.

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